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Estrogens play a pivotal role in 50-60% of breast cancer cases amongst females. Estrogen metabolites have been implicated as mediators in the development of estrogen-induced tumors. The catechol estrogens are particularly interesting chemically since they are prone to undergo further oxidative metabolism and form reactive intermediates like quinones, semiquinones and arene oxides. These reactive intermediates can undergo redox cycling in presence of the metal ions associated with cellular DNA and thereby produce a variety of Reactive Oxygen Species (ROS) like hydoxy, peroxy radicals and superoxide ions. The ROS produce cytotoxic and genotoxic effects in cells and tissues. Recently, 2-methoxyestradiol has been reported as a potent angiogenesis inhibitor via inhibition of tubulin polymerization. Thus, understanding the role of estradiol and its metabolites in estrogen-induced tumorigenesis will provide additional approaches to identify new targets for the development of therapies for prevention and treatment of hormone-dependent breast cancer. This predoctoral fellowship proposal focuses on interdisciplinary approaches to understanding the role of estrogen molecules in cancer development and the structural features critical for tumorigenesis. Chemical efforts are concentrating on the synthesis of catechol estrogens, homologs/mimics of catechol estrogens, and novel estrogen derivatives for probing the oxidative damage hypothesis. Biochemical approaches include the examination of estrogen metabolite formation in cell cultures, *in vitro* measurements of oxidative damage, and effects on tubulin polymerization.

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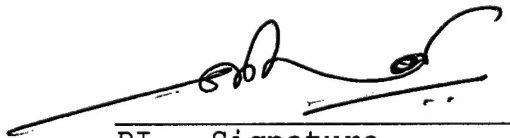

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INTRODUCTION

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Currently, one out of nine American women will develop breast cancer in her lifetime. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, with these cancers characterized as containing estrogen receptors and requiring estrogen for tumor growth.[1] The possible biochemical roles of estrogens in the development of breast cancer remain to be fully elucidated.

Epidemiological studies have shown that women with breast cancer have higher estrogen levels than healthy control women and that estrogen levels are higher in populations characterized by high breast cancer rates.[2] An estimated 60-70% of human breast cancers are associated with sex hormone exposure. The fact that an early menarche and a late menopause are important risk factors for breast cancer suggests a role of the female sex hormones in the etiology of the disease.[3] Also, studies in experimental animals have shown estrogens to induce tumors in hormone-responsive tissues like mammary tissue, uterus, cervix and pituitary.[4] Although estrogens have been implicated as carcinogens, the exact biochemical mechanisms by which estrogens may be tumorigenic remain to be established.

Catechol estrogens, oxidative metabolites of estrogens, have been suggested as possible causative agents in estrogen-induced tumorigenesis. Estrogens are converted to 2-hydroxy and 4-hydroxy derivatives by cytochrome P-450 hydroxylases.[5] Both 2-hydroxyestradiol and 4-hydroxyestradiol have weaker affinity for the estrogen receptor than estradiol and exhibit significantly lower estrogenic activity *in vivo*.[5] However, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones and arene oxides.[6-7] These highly reactive moieties may be cytotoxic via reaction with proteins

and nucleic acids.[8-9] Furthermore, the catechol estrogens have been shown to produce a variety of reactive oxygen species (ROS), such as the hydroxide, peroxide, and superoxide radicals.[10,11,12] These ROS have shown cytotoxic and genotoxic effects in several independent studies.[10,13-14]

Contrasting reports exist in the literature in regard to the tumorigenic potential of 2-hydroxyestradiols vs. 4-hydroxyestradiols. Liehr *et al.* recently reported that microsomes prepared from human mammary adenocarcinoma and fibroadenoma have predominantly 4-hydroxylase activity, suggesting a mechanistic role of 4-hydroxyestradiol in tumor formation.[15] An earlier report demonstrated that 4-hydroxyestradiol formation is predominant in tissues susceptible to estrogen-induced tumorigenesis like Syrian hamster kidney and rat pituitary, whereas 2-hydroxyestradiol formation is predominant in rodent livers where tumors are not produced under similar conditions.[16-18] In contrast, Li and Trush found that 2-hydroxyestradiol produced oxidative damage and strand breaks of double-stranded DNA in the presence of micromolar concentrations of Cu(II), whereas 4-hydroxyestradiol failed to produce any DNA damage.[13,19]

In order to investigate the role of estrogen metabolites in tumor initiation and progression, we have designed, prepared and reported on a series of 2-hydroxyalkyl derivatives.[20] The receptor binding and gene expression potential of these synthetic analogs closely parallels that of 2-hydroxyestradiol. Additionally, these compounds are not able to undergo oxidative metabolism at the 2-position. As a continuing part of this study, we have now prepared the corresponding 4-hydroxyalkylestradiols **1 - 3**. These compounds were designed to provide 4-hydroxysubstituted estrogens that are not able to undergo further oxidative metabolism. On the other hand, compounds **1 - 3** do contain hydroxyl groups at the 3 and 4 positions that are available for hydrogen bonding during protein interactions with receptors and/or enzymes. The

4-aminoalkyl estrogens, compounds **4 - 6** were also synthesized from the hydroxyalkyl derivatives to further elucidate electronic factors at the C-4 position that influence biological activity. Therefore, these analogs may prove useful as chemical probes for differentiating receptor-mediated vs. redox-mediated events in estrogen-induced tumorigenesis. This report describes the synthesis, estrogen receptor affinity and pS2 gene expression studies on the above series of compounds. These compounds were used along with catechol estrogens in *in vitro* studies using calf thymus DNA and evaluated for their ability to induce oxidative DNA damage. Our research group is interested in evaluating the biochemical effects of catechol estrogens, so we needed large amounts of both 2- and 4-hydroxy estradiols. The existing literaure methods for the synthesis of these compounds are not adequate. We have made attempts to improve upon the synthesis of these compounds and the results from these synthetic experiments are reported. The putative role of catechol estrognes in tumor initiation is becasue of thie redox cycling potential. However, on extennsive literature perusal we did not find any reports wherein the redox potential of the catechol estrogens was measured in an aqueous environment. We studied the redox behaviour of catechol estrogens and their analogs using a cyclic voltammeter, the results from that study are reported herein.

BODY

A) Chemistry:

1) Synthesis of 4-Hydroxyalkyl and 4-Aminoalkyl Estradiols:

In our earlier work, the 2-hydroxyalkyl estradiols were prepared via homologation of a protected 2-formylestradiol **7**. [20,21] Pert and Ridley have previously demonstrated that the analogous 4-formylestradiol **8** could be prepared from **10** by lithium-halogen exchange and subsequent reaction of the organolithium with DMF.[22] Unlike the preparation of **7**, wherein yields in excess of 80% were routinely realized, only modest yields of **8** could be obtained. As this synthetic intermediate would be required in large quantities, the homologation of **8** was not considered to be the optimal route available for the preparation of **1-3**. Alternatively, the bisMOM protected 4-bromoestradiol **10** was envisioned to be a suitable partner for a Stille cross-coupling reaction.[23] Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives respectively after hydroboration and oxidation.

The synthesis commenced by brominating estradiol with N-bromosuccinimide in ethanol (Appendix 1, Scheme 1), from which the required 4-bromoestradiol **9** precipitated and was obtained in 54% yield after recrystallization. The bromoestradiol was protected in 75% yield as its bisMOM ether **10** with chloromethyl methyl ether, di-isopropyl ethyl amine in THF at reflux.[22] Using vinyl tributyltin as the alkenyl donor, exploratory experiments were performed to determine the optimal reaction conditions required for the cross-coupling reaction. Thus, reaction of **10** with tetrakistriphenylphosphine palladium(0) (0.06 mol. eq.) and vinyl tributyltin (2.1 mol. eq.) in dry deoxygenated DMF afforded the desired 4-vinyl bisMOM estradiol **11** in 90% yield after heating at reflux overnight. Under similar reaction

conditions, **10** was treated with allyl tributyltin, affording 4-allyl bisMOM estradiol **12** in 94% yield. Using well established chemistry, the unsaturated estradiols **11** and **12** were converted into alcohols **2** and **3**. Thus, hydroboration of **11** with BH_3•THF, followed by oxidative work-up of the alkyl borane with basic hydrogen peroxide, gave the desired alcohol **13**. The allyl estradiol **12** was transformed into **14** in a similar fashion in 75% yield. Subsequent treatment of alcohols **13** or **14** with pyridinium p-toluene sulfonate (PPTS) gave the targeted triols in 70% and 61% yields respectively.

An attempt was made to prepare the 4-formylestradiol **8** by way of a Stille-like reductive-carbonylation as a prelude to preparing alcohol **1**. Treatment of **10** with carbon monoxide, tributyltin hydride and tetrakistriphenylphosphine palladium(0) in DMF at reflux failed to yield **8**. A control reaction in which **8**, prepared by the Pert and Ridley method, was heated for several hours in refluxing DMF demonstrated that it was thermally labile.[22] Indeed, a sample of **8** deteriorated simply on standing at room temperature for a few days.

In view of the instability of **8**, alternate routes for the preparation of other related derivatives were developed. Attempts have been made by Pert and Ridley to introduce an ester group by trapping the organolithium, generated from **10** and n-BuLi with alkyl chloroformates; these reactions were unsuccessful. Treatment of **10** with organolithium (*vide supra*) and carbon dioxide, followed by acidification and subsequent esterification with diazomethane, yielded the methyl ester **15** in 76% yield (Appendix1, Scheme 2). The MOM protecting groups were removed using PPTS in methanol at reflux in 88% yield. Subsequent reduction of the ester **16** with lithium aluminum hydride gave the benzyl alcohol **1** in 51% yield.

The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported.[20,21] Treatment of the bisMOM protected 4-hydroxylalkyl estradiols (**13,14,17**) with phthalimide under Mitsunobu

conditions using triphenylphosphine (PPh_3) and diethyl azodicarboxylate (DEAD) yielded derivatives **18-20** in 70-80% yield (Appendix1, Scheme 3). Subsequent hydrazinolysis in refluxing ethanol gave the bisMOM protected aminoestradiols which, upon treatment with methanolic HCl, gave the desired 4-aminoalkylestradiols **4-6** in good yields.

2) Synthesis of Catechol Estrogens:

Catechol estrogens are chemically unstable and easily decompose by air oxidation. The chemical instability makes catechol estrogens difficult to synthesize. The first reported procedure for synthesizing catechol estrogens involves inverse oxidation of amino estrogens via sodium meta periodate in acetic acid.[25] This method worked reasonably well for making catechol estrones, however we were not able apply it to synthesize 2- and 4-hydroxy estradiols. Zhao *et.al* have employed Dakin oxidation of 2-Acetyl estradiol to give 2-hydroxy estradiol, however this procedure failed to give the desired catechol in our hands.[26] A report by Cushman *et.al* showed that bis-benzyl protected 2-formyl estradiol can be converted into a bis-benzyl protected 2-hydroxy estradiol.[27] We synthesized the bis benzyl protected 2-formyl estradiol using chemistry previously developed in our labs (Appendix 1, Scheme 4). Thus **7** when stirred with 6M HCl for 3 hours in THF gave 2-formyl estradiol **21** in in 98% yield. The formyl estradiol was protected in 85% yield to its bis-benzyl ether **22** with benzyl bromide in DMF. Treatment of this formyl derivative with MCPBA in dichoromethane at $0^{o}C$ yielded the 2-hydroxy bis-benzyl estradiol **23**. Catalytic hydrogenation with 5%Pd-C in ethanol/THF provided the 2-hydroxy estradiol in >80% yield. The main advantage of this method over literature procedures is that by our method the catechol is produced under a reducing environment. This eliminates the oxidative side reactions which occur when the catechols are produced under oxidative conditions reported in literature. The crude 2-hydroxy estradiol was purified using preparative HPLC with 40% CH_3CN/H_2O.

In order to synthesize 4-hydroxy estradiol by employing similiar strategy we needed to synthesize 4-formyl estradiol. There are no good literature procedures for synthesing this compound. We decided to employ a Stille coupling reaction followed by ozonolysis to prepare the 4-formyl estradiol derivative (Appendix 1, Scheme 5). Accordingly, bis-benzyl protected 4-bromo estradiol **24** and a vinyl stannane were refluxed in DMF in presence of $Pd(PPh_3)_4$ to yield the 4-vinyl bis-benzyl protected estradiol **25** in excellent yield. The vinyl compound **25** was subjected to ozonolysis in dichoromethane-MeOH at 0^oC and the reulting ozonide was reduced with dimethyl sulfide to give the benzyl protected 4-formyl estradiol **26**. Baeyer-Villiger oxidation and reduction yielded the 4-hydroxyestradiol in a good yield.

The above reaction sequences are being currently optimized in our labs. Briefly, reaction conditions are being explored under which the formyl estrogens can be synthesized in a single step from the corresponding halo compounds by Pd-catalyzed carbon monoxide insertion. In the initial series of experiments we have realized that bromo estrogens are not suitable coupling partners for CO insertion, instead iodo estrogens may be better coupling partners. Experiments are underway to identify regiospecific iodination conditions.

3) Synthesis of methyl ethers of 2- and 4-hydroxyalkyl estradiols

We have synthesized the methyl ether of 2-hydroxymethyl estradiol as an analog of 2-methoxy estradiol. The stille cross coupling reaction was utilized for this purpose wherein the halo estrogen was refluxed with methoxymethyl tributyl stannane to yield the desired products (Apppendix 1, Scheme 6). however this reaction was not very high yielding and so the methoxymethyl derivative was made by reducing the 2-formyl estrogen **7** with NaBH4, to give the corresponding hydroxymethyl derivative. This was then methylated with methyl iodide in DMSO in presence of powdered KOH.

Synthesis:

General Information.

Estradiol was purchased from Steraloids (Wilton, NH) . All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. Amines were stirred over CaH_2, distilled and then stored over KOH pellets. Silica gel TLC plates (60 F_{254}) were purchased from Analtech Inc (Newark, NE) and visualized with a UV lamp and/or 5% ethanolic phosphomolybdic acid followed by charring. All intermediates were purified by flash column chromatography on silica gel (Merck Kieselgel 60) using the indicated mixtures of hexanes and ethyl acetate. Melting points were determined in open capillaries on a Thomas Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Laser Precision Analytical RFX-40 FTIR Spectrometer in the phase indicated. ^1H NMR and ^{13}C NMR were recorded on an IBM AF/250 spectrometer at 250 and 67.5 MHz respectively in $CDCl_3$ solutions unless otherwise indicated using the residual protiosolvent signal as internal reference. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center on either a VG 70-2505, a Nicolet FTMS-200 or a Finnigan MAT-900 mass spectrometer. Elemental Analysis were performed by Oneida Research Services, Inc (Whitesboro, NY).

<u>NOTE:</u> All the synthetic compounds have been satisfactorily characterized by Elemental analysis and/ or 1H NMR, 13C NMR, Mass spectroscopy. The analysis details have been excluded to maintain brevity of the report. See the attached manuscript for the details on some of the compounds.

4-Bromoestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (10). MOMCl (5.7 mL, 75 mmol) was added dropwise to a cold (0 °C) solution of 4-bromoestradiol (5.26 g, 15.0 mmol) and di-isopropyl ethylamine (21.3 mL, 89.3 mmol) in THF (125 mL).

On completion of the addition, the reaction mixture was allowed to warm up to room temperature, stirred for 1 h at the same temperature, then heated at reflux overnight. The mixture was allowed to cool, then sat. NH_4Cl solution (100 mL) was added. The mixture was extracted with EtOAc (4x100 mL), the combined organic solutions were washed with sat. aqueous brine (100 mL), dried ($MgSO_4$) and concentrated. The crude product was purified by flash column chromatography (SiO_2, hexane/ethyl acetate, 9:1) to afford a pale yellow solid, which was recrystallized from hexane to give 4.78 g (72%) of the desired compound as a colorless solid: mp 88-89 °C (Lit. 97-98 °C)

4-Ethenylestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (11). A solution of **11** (440 mg , 1.0 mmol), vinyl tributyltin (0.62 g, 2.0 mmol) and $Pd(PPh_3)_4$ (67 mg, 0.06 mmol) in DMF (15 mL) was deoxygenated by bubbling argon through it for 15 mins. The solution was heated at reflux overnight, cooled to room temperature and diluted with ether (50 mL), washed with 5% NH_4OH (15 mL), water (4x20 mL), brine (3x20 mL), dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, 4:1 hexane/ethyl acetate) to yield 344 mg (90%) of the title compound as a colorless oil, which solidified on standing to a colorless waxy solid: mp 55 °C.

4-(2'-Propenyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (12). A solution of **11** (1.50 g, 3.41 mmol), $Pd(PPh_3)_4$ (250 mg, 0.22 mmol) and allyl tri-n-butyl stannane (2.28 g, 6.90 mmol) in DMF (50 mL) was deoxygenated by bubbling argon through it for 15 min, then the solution was heated at reflux overnight. After cooling, the solution was decanted off from the palladium, and the residual precipitated palladium was washed with ethyl acetate. The reaction solution was diluted with ethyl acetate, then washed with water (3x50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 10:1) to give 1.28 g (94%) of the desired allyl compound **14** as a colorless oil.

4-(2'-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (13). A solution of 1M BH_3•THF (3.00 mL, 3.00 mmol) was added dropwise to a solution of **12** (286 mg, 0.75 mmol) in THF (6 mL) at 0 °C. On completion of the addition the cooling bath was removed and the mixture stirred for 1 h; 1M NaOH (3 mL) was added cautiously and, after the addition of 30% H_2O_2 (3 mL), the mixture was heated at reflux for 1 h. The mixture was allowed to cool, then ethyl acetate (75 mL) was added and the organic solution was separated from the aqueous layer. The organics were washed with water (25 mL), brine (25 mL), dried ($MgSO_4$) and concentrated. The residue was purified by MPLC (SiO_2, hexane/ethyl acetate, 2:1) to give 37 mg (12%) of a diastereomeric mixture of partially deprotected secondary alcohols, 92 mg (30%) of a mixture of two diastereomeric secondary alcohols and 119 mg (39%) of the desired primary alcohol as a colorless oil, which crystallized on standing: mp 81-82 °C.

4-(3'-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (14). A solution of 1M BH_3•THF (12.4 mL, 12.4 mmol) was added dropwise to a solution of **14** (1.23 g, 3.08 mmol) in THF (25 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 1 h. 1M NaOH (25 mL) was added cautiously, then 30% H_2O_2 (25mL) was added and the resulting mixture heated at reflux for 1 h. The aqueous reaction mixture was extracted with ethyl acetate (3x100 mL) and the organic layer was washed with water (100 mL), brine (100 mL), dried ($MgSO_4$) and concentrated. Column chromatography (SiO_2, hexane/ethyl acetate, 2:1) of the residue gave 1.06 g (82%) of the desired alcohol as a colorless oil.

4-(2'-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol (2). A solution of **13** (80 mg, 0.20 mmol) and pyridinium *p*-toluenesufonate (0.50 g, 2.00 mmol) in methanol (5 mL) were heated at reflux for 24 h. After cooling to room temperature, ethyl acetate (50 mL) was added and

then the solution was washed with water (2x50 mL) and brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was recrystallized from methanol and water to afford 50 mg (80%) of the alcohol: mp 229-230 °C.

4-(3'-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol (3). A solution of the alcohol **14** (173 mg, 0.41 mmol) and pyridinium p-toluenesulfonate (0.50 g, 2.0 mmol) in methanol (5 mL) was heated at reflux for 24h. After cooling, the reaction mixture was diluted with ethyl acetate (50 ml), then it was washed with water (3x25 mL), brine (25 mL), dried and concentrated. The residue was recrystallized from methanol/water to give 61 mg (61%) of the desired alcohol as a colorless solid: mp 240-242 °C.

4-Carboxy-3,17β-Bis(methoxymethoxy)estra-1,3,5(10)-triene 3,17β-diol Methyl Ester (15) n-BuLi (3.5 mL, 5.65 mmol) was added dropwise to a solution of **10** (1.15 g, 2.61 mmol) in THF (50 mL) at -78 °C. After stirring for 1h at this temperature, several pieces of dry-ice were added and then the cooling bath was removed. After warming to room temperature, the reaction mixture was diluted with ether (50 mL) and then extracted with 5% KOH (5x50 mL). The combined basic extracts were acidified to pH 5 with concentrated HCl, then extracted with ether (6x50 mL). The combined ethereal extracts were washed with brine (50 mL), dried ($MgSO_4$) and concentrated to give the crude acid. The acid was suspended in ether (20 mL) and an ethereal solution of diazomethane was added to it at 0 °C. After 30 min., sufficient acetic acid was added to the reaction mixture to discharge the yellow coloration. The reaction mixture was washed with $NaHCO_3$ (2x50 mL), brine (50 mL), dried ($MgSO_4$), concentrated and purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to give 0.82 g (76%) of the desired product as a colorless oil, which crystallized after a few days standing at room temperature: mp 66-68 °C.

4-Carboxy-3,17β-estra-1,3,5(10)-triene 3,17β-diol Methyl Ester (16). A solution of the ester **15** (0.44 g, 1.10 mmol) and pyridinium *p*-toluenesulfonate (2.77 g, 11.0 mmol) in MeOH (10 mL) was heated to reflux for 36 h. After cooling and addition of EtOAc (100 mL), the organic solution was washed with water (2x50 mL), brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was filtered through a short pad of silica gel (ethyl acetate/hexane 1:2) to give 0.30 g (83%) of **16** as a colorless oil, which crystallized on standing: mp 135-136 °C.

4-Hydroxymethylestra-1,3,5(10)-triene 3,17β-diol (1). $LiAlH_4$ (70 mg, 1.89 mmol) was added portionwise to a solution of the ester **16** (51 mg, 0.15 mmol) in THF (5 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 4 h. When the reaction was complete, water (0.07 mL), 15% NaOH (0.07 mL) and water (0.21 mL) were added. Once a granular precipitate had formed, it was removed by suction filtration through Celite, washed with MeOH, and concentrated to yield 23 mg (51%) of the desired alcohol as a colorless solid; mp > 270 °C.

4-Hydroxymethyl-3,17β-bis(methoxymethoxy)estra-1,3,5(10)triene (17). $LiAlH_4$ (340 mg, 9.19 mmol) was added portionwise to a solution of **15** (0.50 g, 1.20 mmol) in THF (30 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature over 1 h, then stirred for 4h. Water (0.34 mL), 15% NaOH (0.34 mL) and water (1.00 mL) were added successively, then the resulting grannular precipitate was removed by filtration through a pad of Celite and $MgSO_4$ (1:1). After concentration of the filtrate it was chromatographed to give 0.43 g (90%) of the desired alcohol as a colorless oil, which slowly crystallized: mp 80-81 °C.

Phthalimides 23-24: General Procedure: DEAD (0.42 mL, 2.40 mmol) was added dropwise to a solution of the alcohol **13, 14,** or **17** (0.80 mmol), phthalimide (0.35 g, 2.40 mmol) and triphenylphosphine (0.63 g, 2.40 mmol) in THF (10 mL) at room temperature, then stirred

overnight. The solvent was removed *in vacuo* then the residue was dissolved in EtOAc (100 mL) and washed with 5% aqueous KOH (4x50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to afford the substituted phthalimides **18-20** as colorless or pale yellow oils, which solidified on standing:

Amines 4-6: General Procedure: A solution of the phthalimides **18-20** (0.46 mmol) and hydrazine (1 mL) in ethanol (10 mL) were heated at reflux for 1h. After cooling, ethyl acetate (50 mL) was added and then the mixture was washed with 5% KOH solution (3x25 mL), brine (25 mL), dried ($MgSO_4$) and concentrated. The residue was dissolved in methanol (5 mL), cooled to 0 °C and then HCl was bubbled through it for 15 min. The cooling bath was removed and then stirring was continued for 3h. The reaction mixture was concentrated, then redissolved in methanol and the resulting green solution was decolorized with charcoal. After filtration and concentration the residue was taken up in the minimum amount of methanol and the product was precipitated out by the addition of ether, affording the amine salts as colorless or yellow solids.

2-Formylestra-1,3,5(10)-triene 3,17β-diol (21). A solution of **7** (2.00g, 5.15mmol), 6M Hcl (25 mL) and THF (25 mL) were stirred at room temperaturefor 3h. The reaction was poured into water and then was extracted with EtOAc (4x50 mL). The combined organics were dried ($MgSO_4$) , concentrated and then chromatographed (SiO2, hexane/ethyl acetate, 2:1) to afford title compound 1.52 g (98%).

3,17-Bis(benzyloxy)estra-1,3,5(10)-triene-2-carbaldehyde (22). NaH (10eq, 912mg, 39 mmol) was added in small portions under argon to a cold (0^oC) solution of **21** (1.14g, 3.8 mmol) in DMF (25 mL). Resulting dirty brown suspension was stirred at same temperature

for 10mins a which distilled benzyl bromide (7.1eq, 3.2 mL) along with catalytic amount of TBAI was added. The solution was stirred at room temperature for 7 hours. Reaction mixture was cooled in an ie bath and quenched with 50% EtOH, pH adjusted to 5, extracted with ether (3 x 100 mL). The organics were washed with water (2x 50 mL) , brine (2 x 50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO2, Hexane/Ether. 5:1). to give 1.45g (65%) of **22**.

3,17-Bis(benzyloxy)-2-hydroxy-estra-1,3,5(10)-triene (23). A solutioin of MCPBA(1.6 mmol of 71%) in dichloromethane (7 mL) was cannulated into a stirring solution of of **22** (650 mg, 1.35 mmol), pTSOH(5 mg) in DMF (5 mL) at room temperature. the solution was stirred for 3 hours, after which the reaction was complete. Excess peracid was destroyed by adding Na2SO3 (15 mL), and sirrring for 30 mins. The product extracted with CH2Cl2 (2 x 25). The organics were washed with water (2 x 20mL) , brine (2 x 50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO2, Hexane/Ether 4:1). to give **23** (65%).

Estra-1,3,5(10)-triene-2,3,17-triol (24). A solutioin of **23** (200mg, 1.35 mmol) in THF-EtOH (1:2) was hydrogenated with 10% Pd-C at 45 psi of hydrogen. The catalyst was separated by centrifugation and the organic concentrated, reidue extracted in ethylacetate. the organic was then washed with water (2 x 50 mL) and concentrated to give crude 2-hydroxy estradiol in 80% yield. The crude product can be further purified by reverse phase HPLC.

NOTE: The compounds in Scheme 5 were prepared in analogous fashion to those in Appendix 1, Scheme 4, yields are as shown in the scheme. Compound **25** was prepared from **24** in an

analogous fashion to the synthesis of **11** from **10** (Appendix 1, Scheme 1) Synthesis of 26 is reported below:

3,17-Bis(benzyloxy)-4 -carbaldehyde -estra-1,3,5(10)-triene (26). A solution of **25** in $MeOH/CH_2CL_2$ (1:3) was cooled to -78°C, Ozone gas passed through the solution till the solutioin turned blue. The reaction was purged with argon for 20 minutes after which DMS(o.1 mL, 5 eq) was addedand coling bath removedwarmed to room temperature and stirred at same for 10 hours. The reaction mixture was concentrated, taken up in EtOAc, Washed with brine, dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO2, Hexane/Ether 4:1). to give **26** (82 %).

B) <u>Biochemistry:</u>

1) Receptor affinity and gene expression studies:

The affinities of the synthetic 4-hydroxyestradiol analogs for the estrogen receptor were assessed in whole cell estrogen receptor binding assays using MCF-7 human mammary cancer cells.[18] The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations.[14] In addition, the cellular uptake and stability of analogs in the whole cell assay can be assessed. The EC_{50} value for estradiol binding to the estrogen receptor in these whole cell assays was found to be 0.180 nM (Appendix 2, Table 1). The synthetic hydroxyestrogen analog with the highest estrogen receptor affinity was 4-hydroxymethylestradiol **1,** exhibiting an EC_{50} value of 364 nM. Overall, the

4-substituted estradiol homologs exhibited significantly weaker affinity for the estrogen receptor

than estradiol, with relative binding affinities (RBA; estradiol = 100) ranging from 0.49 for compound **1** to 0.05 for compound **3** (Appendix 2, Table 1, Fig. 1).

The relative estrogenic activities of the 4-hydroxyestradiol analogs were evaluated by examining the abilities of the synthetic compounds to induce estrogen-dependent gene expression in human breast cancer cells. In human MCF-7 mammary carcinoma cells, the induction of transcription of the pS2 gene is a primary response to estrogen.[22] The induction of pS2 mRNA expression by estradiol, 4-hydroxyestradiol, and 4-hydroxyalkylestrogen analogs **1** - **3** was determined by RNA dot blot analysis.[21] The EC_{50} value for estradiol induction of pS2 mRNA was found to be 0.030 nM. The estradiol homologs exhibited significantly weaker activity than estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) ranging from 0.257 for compound **1** to 0.001 for compound **3** (Appendix 2, Table 2, Fig. 2).

The effects of 4-hydroxymethylestradiol **1** on the growth of hormone-dependent MCF-7 breast cancer cells was investigated since the compound exhibited the highest estrogen receptor affinity of the 4-hydroxylalkyl analogs synthesized. This mitogenic activity was determined by measuring [^{3}H]-thymidine incorporation at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 4-Hydroxymethylestradiol did not affect cellular DNA synthesis in these breast cancer cell lines, whereas estradiol at a concentration of 1 nM significantly increased MCF-7 cell growth (Appendix2, Fig. 3).

Experimental

General Information. [2,4,6,7-^{3}H]Estradiol (98.4 Ci/mmol, ^{3}H-E_2) was purchased from Dupont/NEN (Boston, MA) and was used as received. MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and cells were incubated in a humidified CO_2 incubator (Forma model 3052) with 5% CO_2 atmosphere. A modified Eagle's minimum essential medium (MEM) supplemented with essential amino acids (1.5x), vitamins (1.5x), nonessential amino acids (2x)

and l-glutamine (1x) was obtained from Gibco BRL (Long Island, NY) and was used for maintaining the cells. The sterilized liquid medium was prepared by the OSU Comprehensive Cancer Center by dissolving the powder in water containing sodium chloride (0.487 g/L), pyruvic acid (0.11 g/L), sodium bicarbonate (1.5 g/L) and the pH adjusted to 6.8. Fetal calf serum was obtained from Gibco BRL. Steroids were removed from heat-inactivated fetal calf serum by two treatments with dextran-coated charcoal at 57°C. Tissue culture flasks and supplies were obtained from Corning Glass Works (Corning, NY). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 693 (Dupont/NEN) as the counting solution. Probes for RNA dot blot analysis (pS2:ATCC 57137; 36B4:ATCC 65917) were obtained as purified plasmids from the American Type Culture Collection and amplified by PCR for use in hybridization. Primers used were synthesized by OLIGOS, ETC (Wilsonville, OR) and were:

For pS2:	sense	5'-ATC CCT GAC TCG GGG TCG CCT TTG-3'
	antisense	5'-CAA TCT GTG TTG TGA GCC GAG GCA CAG-3'
For 36B4:	sense	5'-AAA CTG CTG CCT CAT ATC CG-3'
	antisense	5'-TTT CAG CAA GTG GGA AGG TG-3'

Probes were labelled by random priming with Klenow fragment. Analysis of the RNA dot blots were performed on a Molecular Dynamics PhosphoImager SI.

Whole Cell Estrogen Receptor Studies:[20] MCF-7 cells were maintained in a similar fashion as described above. Cells from 90-100% confluent cultures were harvested by treatment with 0.01% trypsin solution and the washed cell pellet was divided into 9.4 cm^2 wells on a six well plate at 1.5-2x10^5 cells/well in modified MEM (2-3 mL) containing 10% steroid free fetal calf serum and gentamycin (20 mg/mL). After 12-24 h at 37 °C, the culture media was removed and replaced and with serum-free modified MEM media (888 μL) containing insulin (5.0 mg/L),

transferrin (5.0 mg/L), glutamine (2 mM) and albumin (2.0 mg/mL). After 48 hours, the media was removed, fresh serum-free modified MEM media added, and the synthetic estrogen **1 - 6** at various concentrations ($3x10^{-5}$ to $1x10^{-5}$ M, 100 μL) were added and incubated for 10 min at 37 °C. To determine total binding, [^{3}H]-estradiol (3.0 nM, 1.0 μCi) was added and the plates were then incubated for 1 h at 37°C. The cells were washed twice with PBS at 4 °C then 95% ethanol (1 mL) was added, followed by standing for 30 min. at room temperature. An aliquot (500 μL) of the ethanol solution was added to Formula 963 and counted on a liquid scintillation counter. The blank samples with no cells and nonspecific binding samples, containing 6 μM unlabeled estradiol, were performed in a comparable manner. Specific binding of [^{3}H]-estradiol was calculated by subtracting the nonspecific binding data from total binding data. The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [^{3}H]-estradiol binding and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

pS2 Induction: MCF-7 cells were maintained in a similar fashion as described above. Cells were plated at a concentration of 5.5 x 10^5 cells/25 cm^2 flask. After 2 days of growth, the cells were rinsed with Ca^{++}, Mg^{++} free PBS and placed on defined media for 48 hours. Defined media contained DMEM/F12 media (Gibco BRL) supplemented with human albumin (2.0 mg/ml), transferrin (5.0 mg/L), bovine insulin (5.0 mg/L), and *l*-glutamine (2 mM). After addition of fresh defined media, the cells were dosed with compound (10^{-10} M to 10^{-5} M), 10nM 17β-estradiol (Sigma, St. Louis, MO) or carrier (95% ethanol). Each compound was tested in triplicate. After 24 hours, total cellular RNA was isolated by an adaptation of the method of Chomczynski and Sacchi.[20] The cells were lysed with a 4M guanidine isothiocyanate solution, and the lysate acidified with 3M sodium acetate, pH 5.2 (1:10 vol). After addition of 3M NaOAc, pH 5.2 (1:10 vol.), RNA was extracted twice using water saturated phenol:chloroform:isoamyl alcohol (60:24:1) at pH 4.0. A final extraction using an equal volume of chloroform:isoamyl alcohol (25:1) was performed. RNA from the resulting

aqueous layer was precipitated with an equal volume of isopropanol at -20°C for one hour. The RNA was pelleted at 15,000xg for 30 minutes at 4° C. The resulting pellet was washed 2X with 70% ethanol and 1X with 95% ethanol. Dried pellets were resuspended in 30*ul* of Dnase-, Rnase- free molecular biology grade water (Sigma Chemical Co.). Quantification of RNA in each sample was performed using the absorbance at 260nm.

Dot Blot Analysis: A denaturing solution containing 50% formamide, 7% formaldehyde, and 1X SSPE was added to 15 μg of RNA from each sample. The RNA was denatured at 68°C for 15 minutes. Two volumes of 10X SSPE was added to each sample. The samples were loaded onto a 0.45 μm, positively charged, nylon membrane (Schleicher and Schuell, Keene, NH) using gentle suction through a 96-well dot blot manifold (BioRad, Hercules, CA). Membranes probed for pS2 gene expression were loaded with 10 μg RNA, the remaining 5 μg was loaded onto a membrane probed for the control gene, 36B4. Membranes were baked at 80° C for 1 hour and then incubated for at least 3 hours in a pre-hydridization solution containing 5X SSPE, 5X Denhardts Reagent, 2% SDS, 100 ug/ml salmon sperm DNA and 50% formamide. pS2 and 36B4 cDNA was prepared as described above and used to make ^{32}P-radiolabelled probes using random primers in the RadPrime Kit (Gibco BRL). Probes with specific activity ranging from 5.0 x 10^5 to 2.0 x 10^6 cpm/ng were used. Membranes, probed separately for pS2 or 36B4, were incubated for 48 hours or 24 hours, respectively, in hybridization solution containing 5X SSPE, 5X Denhardts reagent, 1% SDS, 100 ug/ml salmon sperm DNA, 10% PEG, and 50% formamide. The membranes were washed in: 0.5X SSPE, 60', at 55°C; 0.1X SSPE, 60', 60°C; and 0.1X SSPE, 60', 65°C. Phosphor screens were exposed for at least one hour and scanned on the PhosphorImager SI (Molecular Dynamics). Quantification of the signal was performed using ImageQuaNT™ software (Molecular Dynamics). The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal induction of pS2 mRNA and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

Cell Growth Assay: Human mammary carcinoma cell lines were maintained in 75-cm^2 plastic flasks at 37°C in a modified Eagle's MEM (10 ml) containing 10% fetal calf serum and gentamycin (20 mg/l). For determination of the effects of the synthetic 4-hydroxymethylestradiol **1** on cell growth, the mammary carcinoma cells were divided into 9.4 cm^2 wells at approximately 100,000 cells/well in modified MEM (2 ml) containing 10% steroid-free fetal calf serum and gentamycin (20 mg/l). After two days, media was changed to serum-free modified MEM and experiments initiated. Cell number was determined by measuring the DNA content of the cultures using the diphenylamine assay (25). Effects on cell division were measured by the addition of [^{3}H]-thymidine (1 μCi/well), followed by incubation for 2 hours, cell lysis, and determination of [^{3}H]-thymidine incorporation into DNA. To determine dose-dependent effects, varying concentrations of 4-hydroxymethylestradiol (3 nM to 10 μM in 5 μl 95% ethanol) were added and incubated for 4 days. Each experiment was carried out in quadriplicates, and test compounds were evaluated in experiments performed at least three different times. Statistical differences between control and treated groups were determined using the Student's *t* test.

2) Cyclic Voltammetry (CV) Studies:

The redox cycling behaviour of catechol estrogens and their analogs was studied in aqueous conditions using cyclic voltammeter. The CV studies were performws in aueous medium at physiological pH with a glassy carbon electrode. 2-hydroxy estradiol and 4-hydroxy estradiolhave quasi-reversible reduction potentials at 273 and 274mV against the standard hydrogen electrode (SHE) respectively. Some studies in literature report detection of semiquinone radicals, however no evidence of semiquinone formation was detected at scan rates of 100mV/ s. This is probably because the studies reported in literature are performed in dry, organic solvents wherein radicals stay around long enough to be detected, however in our biomimetic system the semiquinone radical is probably quenched very rapidly (Appendix 3a) .

The catechol estrogen analogs lack the catechol function on the A-ring and so we did not expect to see redox curves with these compounds. The 2- and 4-hydroxymethyl estradiols had one anodic peak at around 600mV v/s SHE electrode. This is probably due to one electron oxidation of phenolic hydroxyl, which is rapidly in aqueous environment (Appendix 3b). We have performed similiar studies on the other analogs, all the results followed our expectations as mentioned in this discussion (data not shown).

3) Oxidative DNA damage:

The chemistry part of this report details the synthesis of metabolically stable analogs of catechol estrogens. We are using these analogs to further understand the receptor mediated and redox mediated events in estrogen induced tumorigenesis. Redox cycling can produce DNA damage resulting in DNA lesions such as 8-oxo-2-deoxyguanosine (8-oxo-2dG). In our initial in vitro studies we exposed calf thymus DNA to varying amounts of catechol estrogens and their non-redox cycling counterparts and measured the elevated levels of 8-oxo-dGas a marker of DNA damage. The DNA was analyzed at specific time intervals using reverse phase HPLC coupled with an electrochemical detector (ECD). The HPLC-ECD system can accurately quantitate 8-oxo-dG in low femtomolar concentration. These studies have shown that both 2- and 4-Hydroxy estradiol produce oxidative DNA damage at the same rate and to the same extent, whereas the non -redox cycling analogs are incapable of producing DNA damage. In another series of studies copper sulfate was added to the incubation in conjunction with estrogens. The amount of 8-oxo-dG was doubled over the earlier study, thus showing the importance of redox active metals in mediating toxicity (Appendix 4).

The calf thymus DNA studies confirm the hypothesis that catechol estrogens , the only redox active estrogens in the series tested were capable of producing oxidative DNA damage. However it should be noted that 10uM or greater concentratiions of catechol estrogens were needed to acquire significant damage.

CONCLUSIONS

The Stille cross-coupling and the carboxymethylation reaction reported here represent two efficient, previously unexplored synthetic routes for the functionalization of the 4-position of estradiol. The synthesis of the 4-hydroxyalkyl estrogens was accomplished by oxidative hydroboration of 4-alkenyl estradiols, which were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenyl stannane. The 4-aminoalkyl estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions. This report also details our attempts to synthesize catechol estrogens by employing a Baeyer-Villiger reaction . This method has distinct advantages over the present literature protocols . We are inthe process of optimising the various steps of the reaction scheme . The synthetic work reported herein enables us to access variously functionalized estrogen molecules, one such example is 2-Methoxymethyl estradiol rreported herein. This compound has displayed interesting biological profile (data not shown), eperiments are ongoing to validate these findings.

The substituted estradiols were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. The 4-hydroxyalkylestradiols had significantly lower affinity for the estrogen receptor when compared with the endogenous ligand, estradiol. 4-Hydroxymethylestradiol (**1**) exhibited the highest affinity of the synthetic compounds, with an apparent EC_{50} value of 364 nM, and it exhibited a similar affinity as the endogenous metabolite, 4-hydroxyestradiol, in the whole cell assays. On the other hand, the 4-aminoalkylestradiols (**4** - **6**) exhibited either extremely weak or no affinity for the estrogen receptor.

Estradiol acts through the nuclear estrogen receptor to induce the transcription of a variety of hormone-responsive genes in target tissues, and induction of pS2 gene transcription is a primary response to estrogen observed in human MCF-7 mammary carcinoma cells.[22] The

4-hydroxyalkylestradiols had significantly decreased efficacy for the induction of pS2 mRNA levels in MCF-7 cells when compared with the endogenous ligand, estradiol. Again, 4-hydroxymethylestradiol (**1**) was the most potent among the synthetic compounds, with an apparent EC_{50} value of 11.7 nM. This synthetic compound was more effective than the endogenous metabolite, 4-hydroxyestradiol, which exhibited an apparent EC_{50} value of 65.4 nM.

Thus, the 4-hydroxyalkyl estradiols **1** - **3** exhibited both significantly weaker estrogen receptor affinities and abilities to induce pS2 gene expression in MCF-7 cell cultures. These results are consistent with the established structure-activity relationships of estrogens and the limitations of A-ring substitutions on the estrogen molecule in producing estrogen receptor-mediated responses. On the other hand, 4-hydroxymethylestradiol (**1**) exhibited similar estrogen receptor affinity and similar induction of pS2 gene transcription as the catechol estrogen, 4-hydroxyestradiol. This catechol estradiol has been implicated as a possible causative agent in estrogen-induced tumorigenesis; however, *in vitro* and *in vivo* investigations with 4-hydroxyestradiol are difficult due to its chemical and biochemical instability. Thus, 4-hydroxymethylestradiol (**1**) may be viewed as a chemically stable catechol estrogen homolog and may therefore prove useful in examination of the role of catechol estrogens in normal physiology and in pathological states, such as estrogen-induced tumorigenesis.

The cyclic voltammetry studies proved that catechol estrogens can undergo redox cycling in aqueous environment, whereas the hydroxyalkyl derivatives are unable to undergo redox cycling. The *in vitro* studies with calf thymus indicated that both 2- and 4- hydroxy estradiols can produce oxidative damage at a similar rate and to the same extent in our system.

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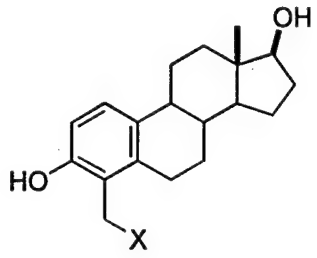
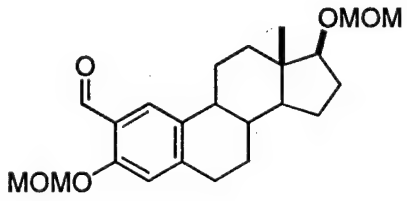
APPENDIX 1

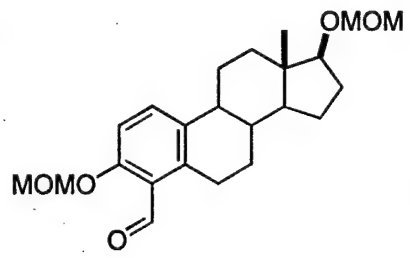
Synthetic Targets and Intermediates



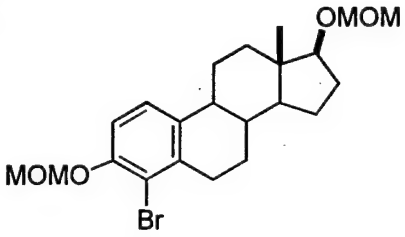
1: X=OH
2: X=CH_2OH
3: X=CH_2CH_2OH
4: X=NH_2
5: X=CH_2NH_2
6: X=$CH_2CH_2NH_2$



7

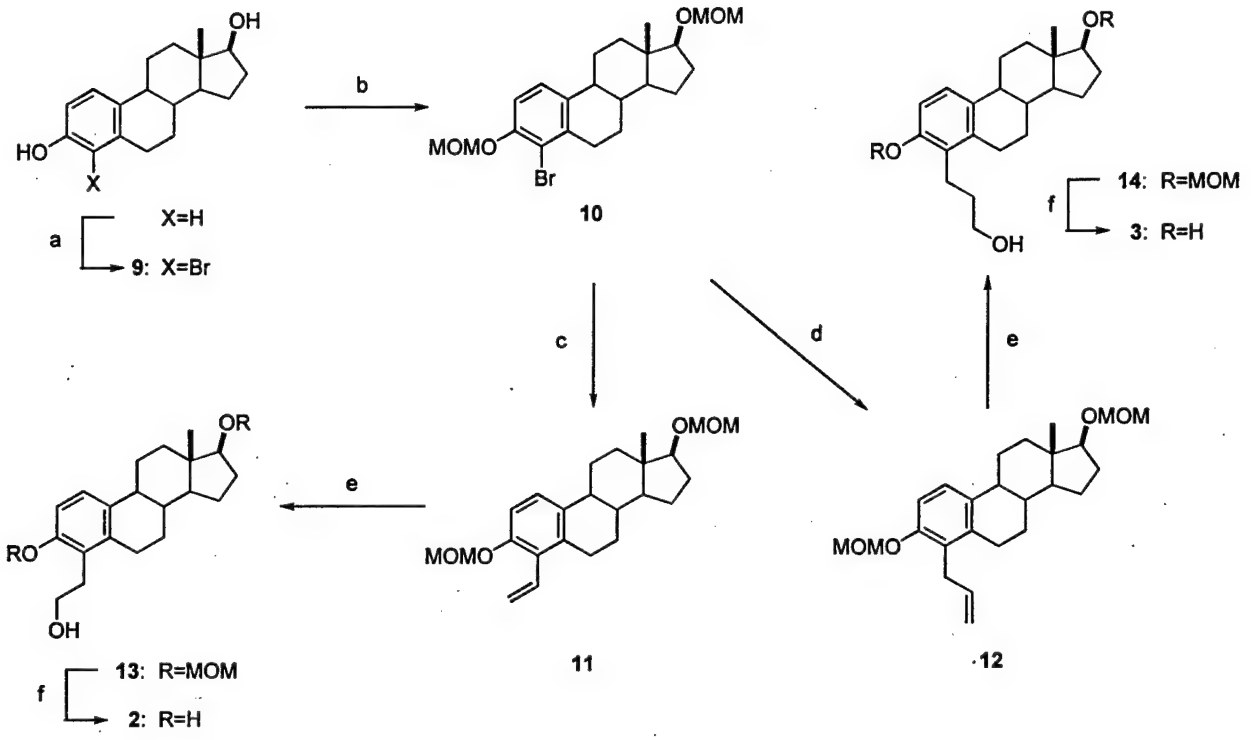


8



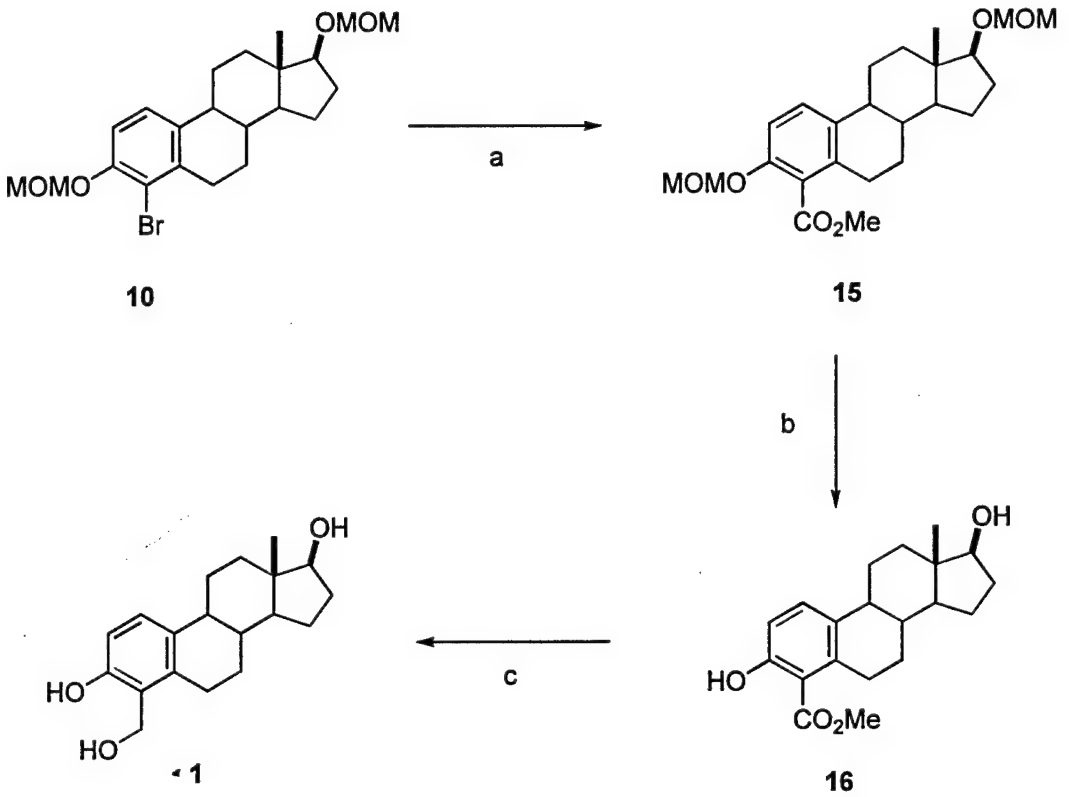
10

Scheme 1



Reagents and conditions: a. N-Bromosuccinimide, EtOH, 54%; b. MOMCl, *i*-Pr_2NEt, THF, Δ, 75%; c. $Pd(PPh_3)_4$, $CH_2{=}CHSnBu_3$, DMF, Δ, 90%; d. $Pd(PPh_3)_4$, $CH_3CH{=}CHSnBu_3$, DMF, Δ, 94%; e. (i) $BH_3{\bullet}THF$, THF, 0 °C, (ii) NaOH, H_2O_2, Δ, **11**→**13** 39%, **12**→**14**, 82%, f. PPTS, MeOH, Δ, **13**→**2** 80%, **14**→**3** 61%.

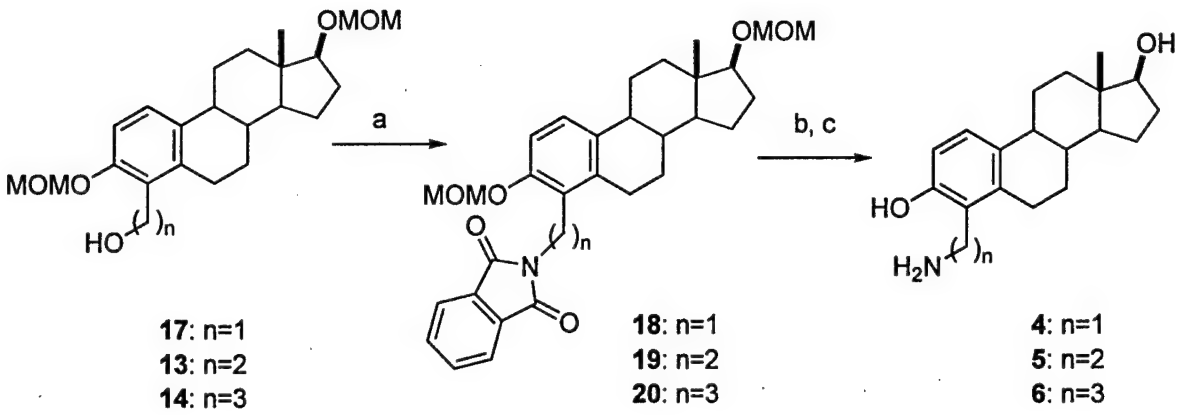
Scheme 2



10 15 16 1

Reagents and conditions: a. (i) *n*-BuLi, THF, -78 °C, (ii) CO_2, -78 °C→RT; (iii) CH_2N_2, Et_2O, 0 °C, 76%, b. PPTS, MeOH, Δ, 83%; c. $LiAlH_4$, THF, 0 °C→RT, 51%.

Scheme 3



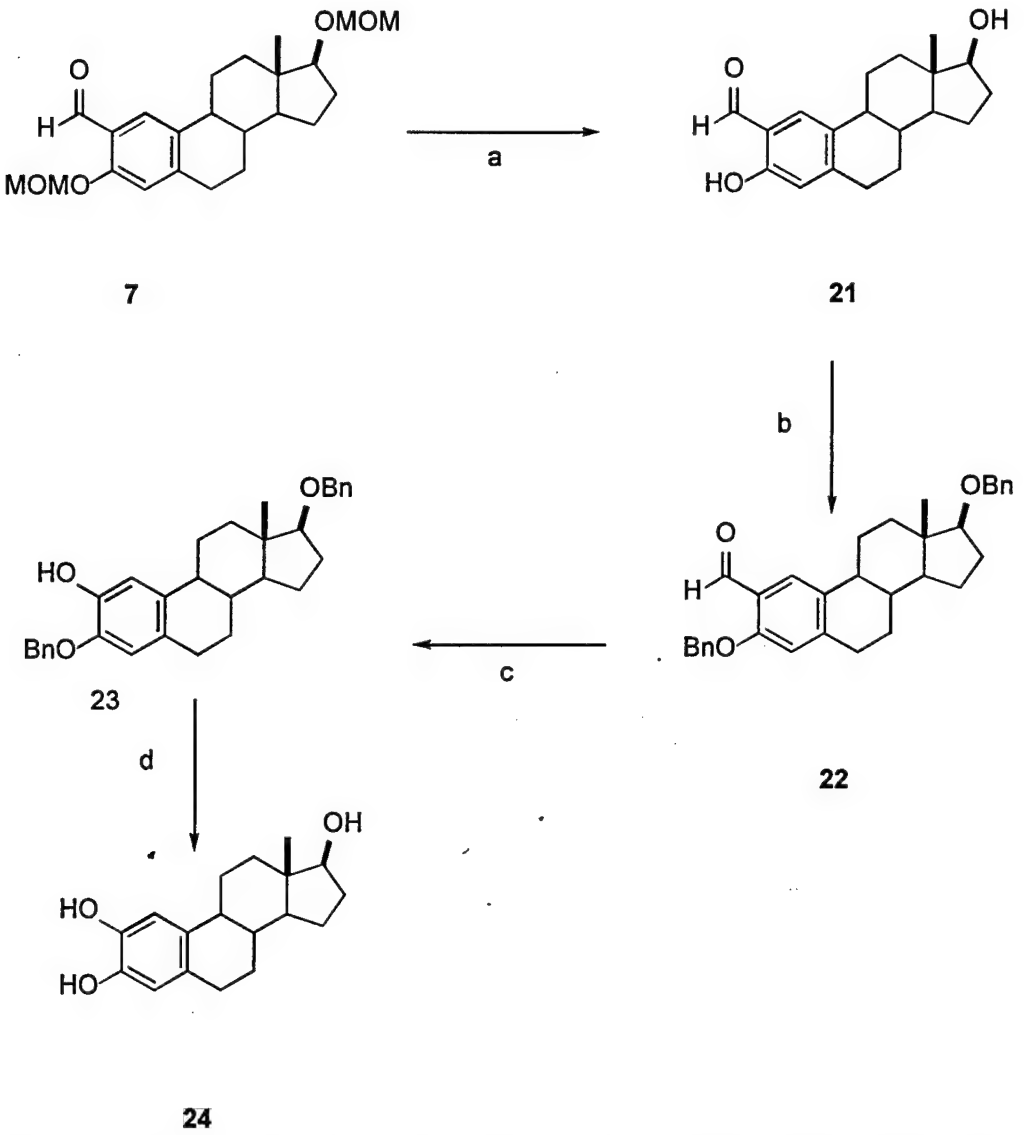
17: n=1
13: n=2
14: n=3

18: n=1
19: n=2
20: n=3

4: n=1
5: n=2
6: n=3

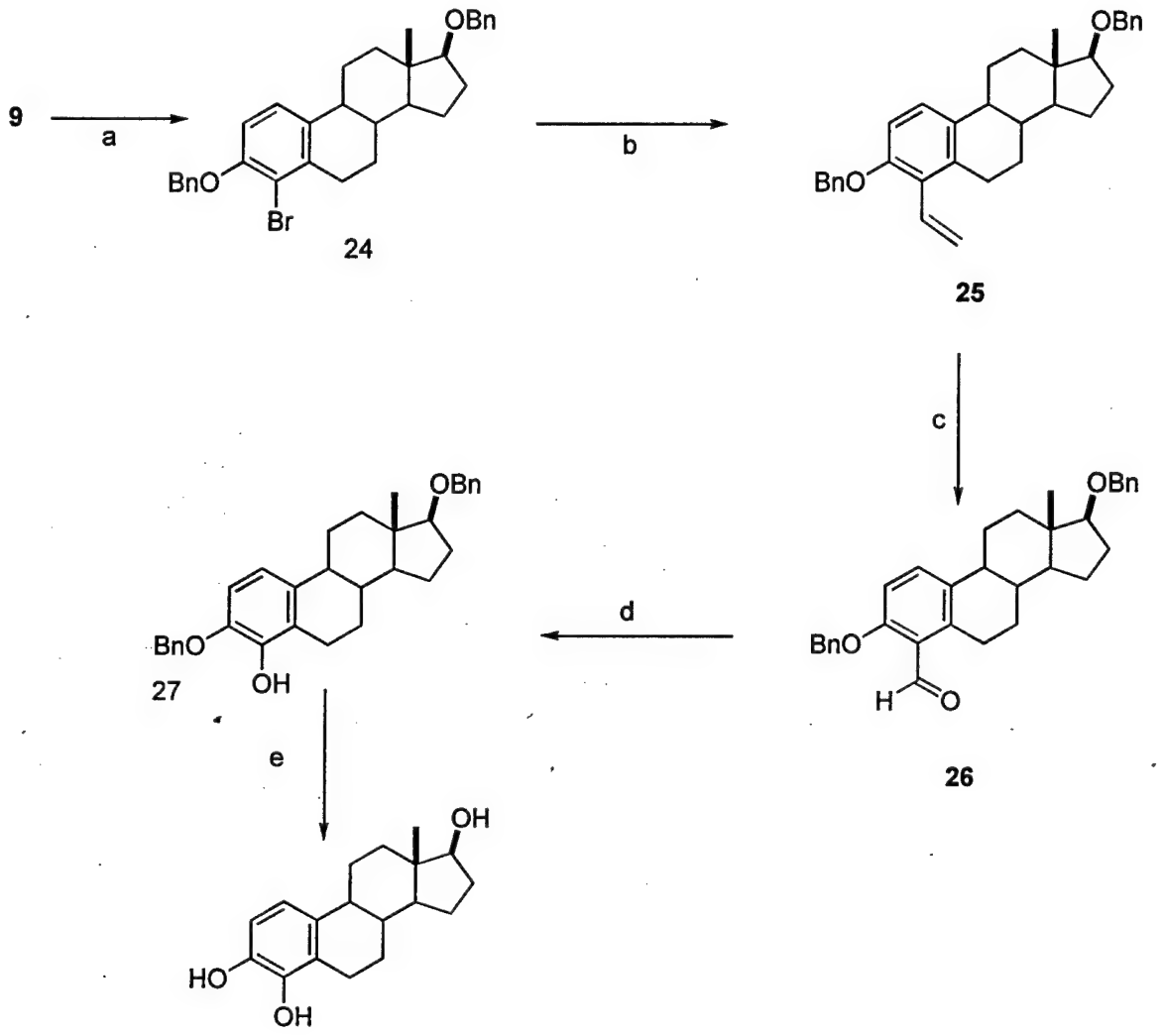
Reagents and conditions: a. PhthNH, DEAD, Ph_3P, THF; b. NH_2NH_2, EtOH, Δ; c. HCl, MeOH.

Scheme 4



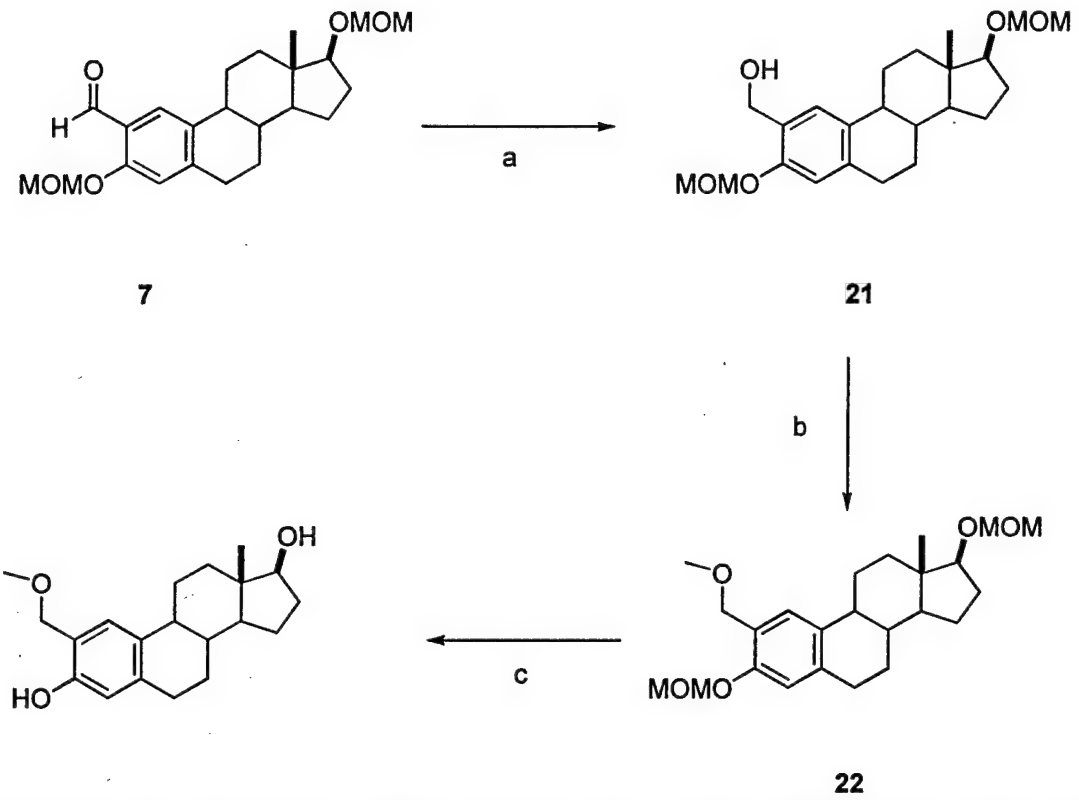
Reagents and conditions: a. 6M HCl, THF, 98%; b. NaH, TBAI, BnBr, DMF, 65%; c.MCPBA, pTSOH, CH_2Cl_2, 73%; d. 10% Pd-C/H_2, 80%.

Scheme 5



Reagents and conditions: a. NaH, TBAI, BnBr, DMF, 80%; b. $Bu_3SnCH{=}CH_2$, $Pd(PPh_3)_4$. c i) O3, -78OC, MeOH-CH2Cl2. ii) DMS d.MCPBA, pTSOH, CH_2Cl_2, 73%; e. 10% Pd-C/H_2, 80%.

Scheme 6



Reagents and conditions: a. $NaBH_4$, MeOH, 90% b.KOH, DMSO, MeI, 69% c. PPTS, MeOH.

APPENDIX 2

Table 1.

Estrogen Receptor Affinity of 4-Substituted Estradiol Analogs

steroid	cpd	EC_{50}	Log EC_{50} ± SD	RBA
estradiol		1.80×10^{-10} M	-9.744 ± 0.102	100.00
4-hydroxyestradiol		5.06×10^{-7} M	-6.295 ± 0.092	0.36
4-hydroxymethylestradiol	**1**	3.64×10^{-7} M	-6.438 ± 0.141	0.49
4-hydroxyethylestradiol	**2**	6.20×10^{-7} M	-6.207 ± 0.290	0.29
4-hydroxypropylestradiol	**3**	3.32×10^{-6} M	-5.479 ± 0.116	0.05
4-aminomethylestradiol	**4**	NB		-
4-aminoethylestradiol	**5**	2.50×10^{-6} M	-5.600 ± 0.159	0.07
4-aminopropylestradiol	**6**	NB		-

NB = no measurable binding of steroid at 10^{-5} M concentration

Table 2.

Induction of pS2 Gene Expression by 4-Substituted Estradiol Analogs

steroid	cpd	EC_{50}	Log EC_{50} ± SD	%Relative Activity
estradiol		3.01×10^{-11} M	-10.520 ± 0.217	100.00
4-hydroxyestradiol		6.54×10^{-8} M	-7.184 ± 0.158	0.046
4-hydroxymethylestradiol	**1**	1.17×10^{-8} M	-7.933 ± 0.288	0.257
4-hydroxyethylestradiol	**2**	1.48×10^{-7} M	-6.829 ± 0.094	0.020
4-hydroxypropylestradiol	**3**	2.95×10^{-6} M	-5.530 ± 0.217	0.001

Figure Legends

Figure 1. Estrogen receptor competitive binding assays for estradiol (■), 4-hydroxy-methylestradiol (●), and 4-hydroxypropylestradiol (□).

Figure 2. Induction of pS2 gene expression by estradiol (●), 4-hydroxyestradiol (▲), 4-hydroxymethylestradiol (□), 4-hydroxyethylestradiol (▽), and 4-hydroxypropylestradiol (◆).

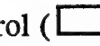
Figure 3. Comparison of mitogenic activities of estradiol (), 4-hydroxymethylestradiol (), and vehicle control () in MCF-7 human mammary carcinoma cell cultures.

Figure 1

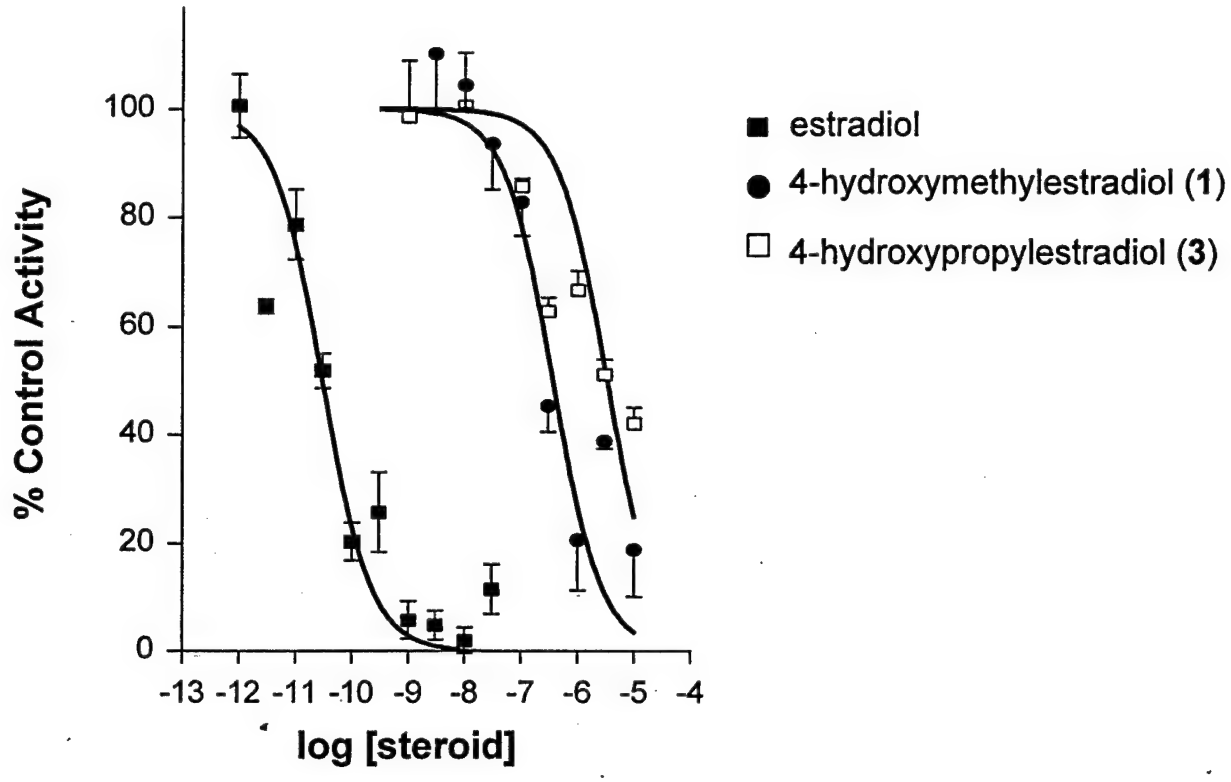

MCF-7 Estrogen Receptor Competitive Binding Assay
% Control Activity
100
80
60
40
20
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log [steroid]
estradiol
4-hydroxymethylestradiol (1)
4-hydroxypropylestradiol (3)

Figure 2

Induction of pS2 Gene Expression

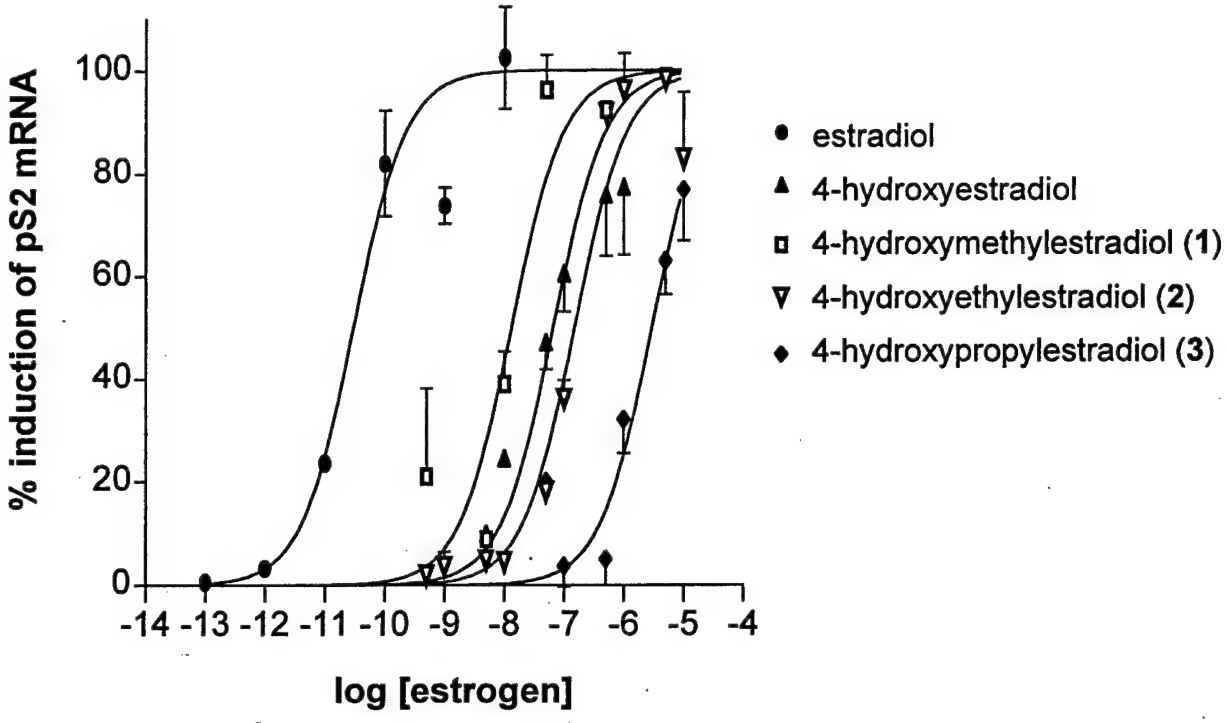
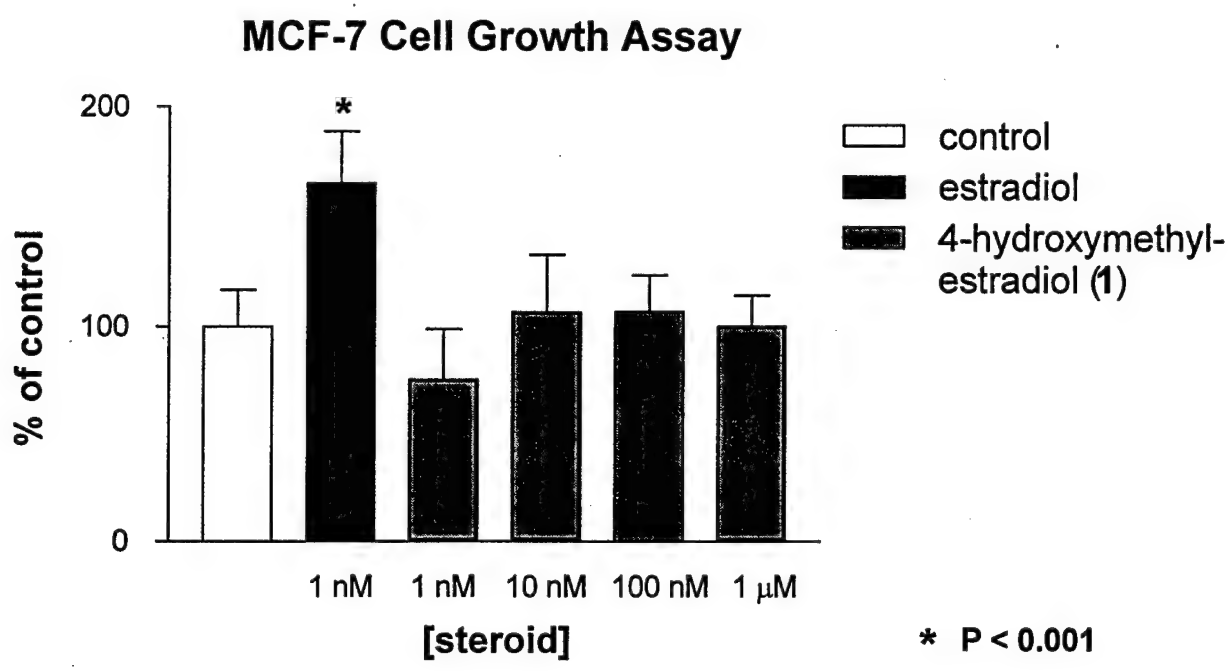
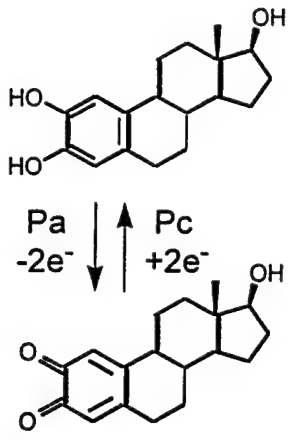


Figure 3


MCF-7 Cell Growth Assay
200
100
0
% of control
*
1 nM
1 nM
10 nM
100 nM
1 μM
[steroid]
control
estradiol
4-hydroxymethyl-estradiol (1)
* P < 0.001

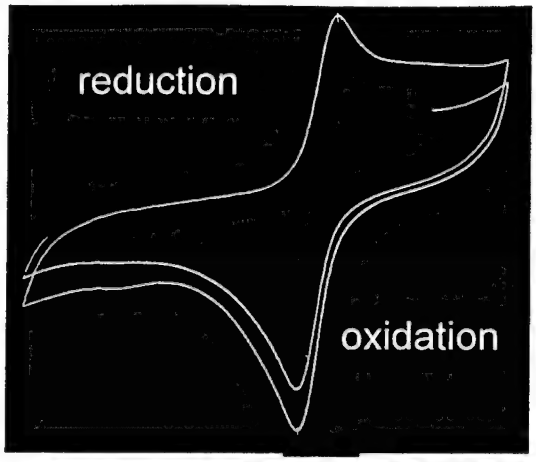
APPENDIX 3

Potentiometric Studies (2-OH-E2)



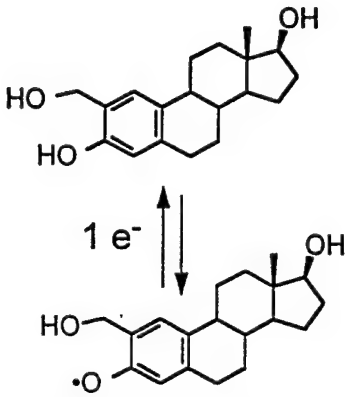
$E_{1/2}$ vs SHE
@ pH 7.4 Phosphate Buffer

Pc = 273



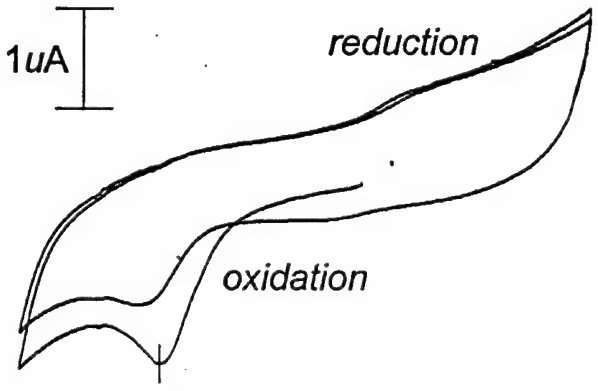
Pa = 274

Potentiometric Studies (2-HMe-E2)



Phenolic Radical

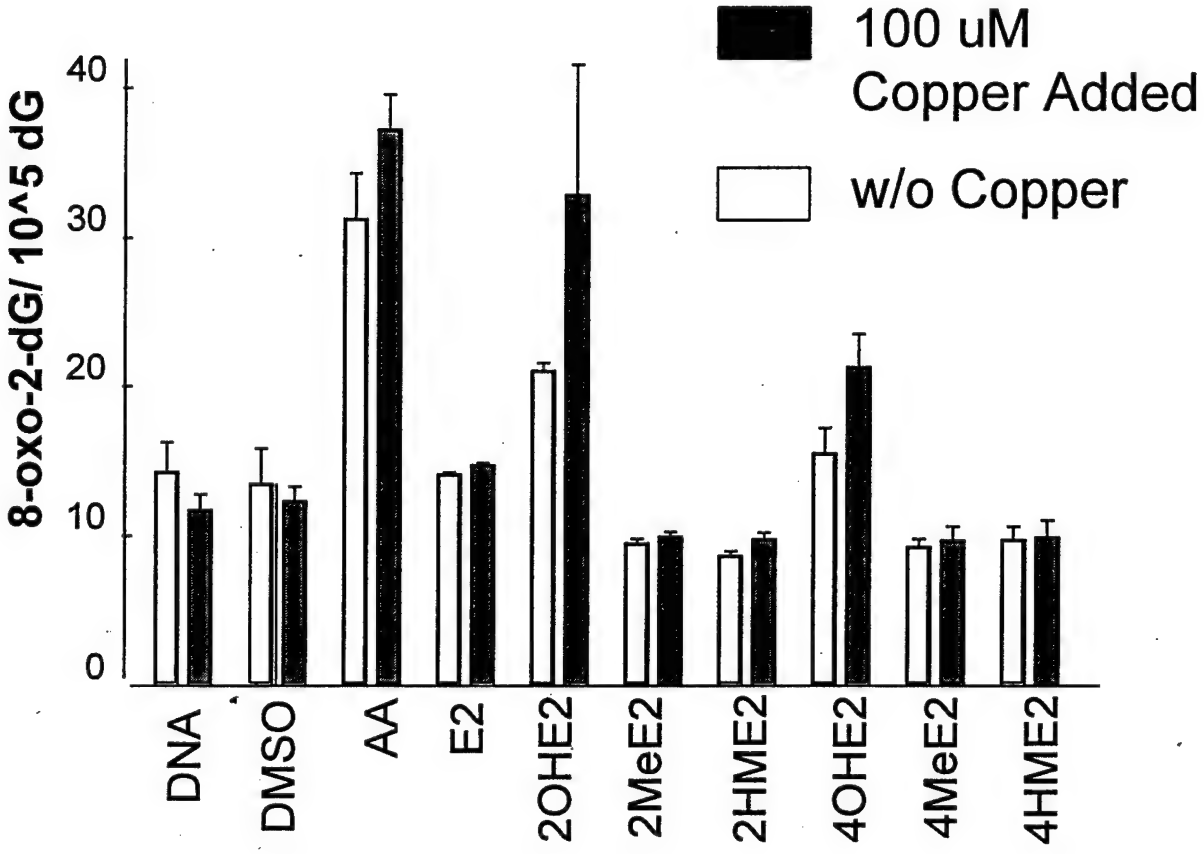
$E_{1/2}$ vs Hydrogen Electrode
@ pH 7.4 phophate buffer



Pa= 597 mV

APPENDIX 4

Results: Calf Thymus DNA (3 Hr. Exposure)



APPENDIX 5

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SYNTHESIS AND BIOLOGICAL EVALUATION OF 4-(HYDROXYALKYL)ESTRADIOLS AND RELATED COMPOUNDS

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ABSTRACT

A series of synthetic estrogens containing hydroxyalkyl side chains at the C-4 position of the A-ring were designed as metabolically stable analogs of 4-hydroxyestradiol, a catechol estrogen. These synthetic steroids would facilitate investigations on the potential biological role of catechol estrogens and also enable further examination of the structural and electronic constraints on the A-ring in the interaction of estrogens with the estrogen receptor. Catechol estrogens are implicated as possible causative agents in estrogen-induced tumorigenesis. 4-Hydroxyestradiol has weaker affinity for the estrogen receptor and exhibits lower estrogenic activity *in vivo*; on the other hand, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates. This report describes the synthesis and initial biochemical evaluation of 4-hydroxyalkyl estrogens and 4-aminoalkyl estradiols. The 4-hydroxyalkyl estrogens were prepared by oxidative hydroboration of 4-alkenyl estradiols. The alkenyl estradiols were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenyl stannane. The 4-aminoalkyl estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions. The substituted estradiols were evaluated for estrogen receptor binding activity in MCF-7 human mammary carcinoma cells, and 4-hydroxymethylestradiol **1** exhibited the highest affinity with an apparent EC_{50} value of 364 nM. The relative activities for mRNA induction of the pS2 gene in MCF-7 cell cultures by the 4-hydroxyalkyl estrogens closely parallel the relative binding affinities. 4-Hydroxymethylestradiol **1** did not stimulate the growth of MCF-7 cells at concentrations up to 1 μM. Thus, 4-hydroxymethylestradiol **1** exhibited similar estrogen receptor affinity as the catechol estrogen, 4-hydroxyestradiol, and may prove useful in the examination of the biological effects of 4-hydroxyestrogens.

INTRODUCTION

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Currently, one out of nine American women will develop breast cancer in her lifetime. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, with these cancers characterized as containing estrogen receptors and requiring estrogen for tumor growth.[1] The possible biochemical roles of estrogens in the development of breast cancer remain to be fully elucidated.

Epidemiological studies have shown that women with breast cancer have higher estrogen levels than healthy control women and that estrogen levels are higher in populations characterized by high breast cancer rates.[2] An estimated 60-70% of human breast cancers are associated with sex hormone exposure. The fact that an early menarche and a late menopause are important risk factors for breast cancer suggests a role of the female sex hormones in the etiology of the disease.[3] Also, studies in experimental animals have shown estrogens to induce tumors in hormone-responsive tissues like mammary tissue, uterus, cervix and pituitary.[4] Although estrogens have been implicated as carcinogens, the exact biochemical mechanisms by which estrogens may be tumorigenic remain to be established.

Catechol estrogens, oxidative metabolites of estrogens, have been suggested as possible causative agents in estrogen-induced tumorigenesis. Estrogens are converted to 2-hydroxy and 4-hydroxy derivatives by cytochrome P-450 hydroxylases.[5] Both 2-hydroxyestradiol and 4-hydroxyestradiol have weaker affinity for the estrogen receptor than estradiol and exhibit significantly lower estrogenic activity *in vivo*.[5] However, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones and arene oxides.[6-7] These highly reactive moieties may be cytotoxic via reaction with proteins and nucleic acids.[8-9] Furthermore, the catechol estrogens have been shown to produce a variety

of reactive oxygen species (ROS), such as the hydroxide, peroxide, and superoxide radicals.[10,11,12] These ROS have shown cytotoxic and genotoxic effects in several independent studies.[10,13-14]

Contrasting reports exist in the literature in regard to the tumorigenic potential of 2-hydroxyestradiols vs. 4-hydroxyestradiols. Liehr *et al.* recently reported that microsomes prepared from human mammary adenocarcinoma and fibroadenoma have predominantly 4-hydroxylase activity, suggesting a mechanistic role of 4-hydroxyestradiol in tumor formation.[15] An earlier report demonstrated that 4-hydroxyestradiol formation is predominant in tissues susceptible to estrogen-induced tumorigenesis like Syrian hamster kidney and rat pituitary, whereas 2-hydroxyestradiol formation is predominant in rodent livers where tumors are not produced under similar conditions.[16-18] In contrast, Li and Trush found that 2-hydroxyestradiol produced oxidative damage and strand breaks of double-stranded DNA in the presence of micromolar concentrations of Cu(II), whereas 4-hydroxyestradiol failed to produce any DNA damage.[13,19]

In order to investigate the role of estrogen metabolites in tumor initiation and progression, we have designed, prepared and reported on a series of 2-hydroxyalkyl derivatives.[20] The receptor binding and gene expression potential of these synthetic analogs closely parallels that of 2-hydroxyestradiol. Additionally, these compounds are not able to undergo oxidative metabolism at the 2-position. As a continuing part of this study, we have now prepared the corresponding 4-hydroxyalkylestradiols **1 - 3**. These compounds were designed to provide 4-hydroxysubstituted estrogens that are not able to undergo further oxidative metabolism. On the other hand, compounds **1 - 3** do contain hydroxyl groups at the 3 and 4 positions that are available for hydrogen bonding during protein interactions with receptors and/or enzymes. The 4-aminoalkyl estrogens, compounds **4 - 6** were also synthesized from the hydroxyalkyl derivatives to further elucidate electronic factors at the C-4 position that influence biological activity. Therefore, these analogs may prove useful as chemical probes

for differentiating receptor-mediated vs. redox-mediated events in estrogen-induced tumorigenesis. The synthesis and initial biochemical evaluation of these 4-hydroxyestradiol metabolite analogs are reported in this paper.

RESULTS AND DISCUSSION

Chemistry

In our earlier work, the 2-hydroxyalkyl estradiols were prepared via homologation of a protected 2-formylestradiol **7**. [20,21] Pert and Ridley have previously demonstrated that the analogous 4-formylestradiol **8** could be prepared from **10** by lithium-halogen exchange and subsequent reaction of the organolithium with DMF.[22] Unlike the preparation of **7**, wherein yields in excess of 80% were routinely realized, only modest yields of **8** could be obtained. As this synthetic intermediate would be required in large quantities, the homologation of **8** was not considered to be the optimal route available for the preparation of **1-3**. Alternatively, the bisMOM protected 4-bromoestradiol **10** was envisioned to be a suitable partner for a Stille cross-coupling reaction.[23] Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives respectively after hydroboration and oxidation.

The synthesis commenced by brominating estradiol with N-bromosuccinimide in ethanol (Scheme 1), from which the required 4-bromoestradiol **9** precipitated and was obtained in 54% yield after recrystallization. The bromoestradiol was protected in 75% yield as its bisMOM ether **10** with chloromethyl methyl ether, di-isopropyl ethyl amine in THF at reflux.[22] Using vinyl tributyltin as the alkenyl donor, exploratory experiments were performed to determine the optimal reaction conditions required for the cross-coupling reaction. Thus, reaction of **10** with tetrakistriphenylphosphine palladium(0) (0.06 mol. eq.) and vinyl

tributyltin (2.1 mol. eq.) in dry deoxygenated DMF afforded the desired 4-vinyl bisMOM estradiol **11** in 90% yield after heating at reflux overnight. Under similar reaction conditions, **10** was treated with allyl tributyltin, affording 4-allyl bisMOM estradiol **12** in 94% yield. Using well established chemistry, the unsaturated estradiols **11** and **12** were converted into alcohols **2** and **3**. Thus, hydroboration of **11** with BH_3•THF, followed by oxidative work-up of the alkyl borane with basic hydrogen peroxide, gave the desired alcohol **13**. The allyl estradiol **12** was transformed into **14** in a similar fashion in 75% yield. Subsequent treatment of alcohols **13** or **14** with pyridinium p-toluene sulfonate (PPTS) gave the targeted triols in 70% and 61% yields respectively.

An attempt was made to prepare the 4-formylestradiol **8** by way of a Stille-like reductive-carbonylation as a prelude to preparing alcohol **1**. Treatment of **10** with carbon monoxide, tributyltin hydride and tetrakistriphenylphosphine palladium(0) in DMF at reflux failed to yield **8**. A control reaction in which **8**, prepared by the Pert and Ridley method, was heated for several hours in refluxing DMF demonstrated that it was thermally labile.[22] Indeed, a sample of **8** deteriorated simply on standing at room temperature for a few days.

In view of the instability of **8**, alternate routes for the preparation of other related derivatives were developed. Attempts have been made by Pert and Ridley to introduce an ester group by trapping the organolithium, generated from **10** and n-BuLi with alkyl chloroformates; these reactions were unsuccessful. Treatment of **10** with organolithium (*vide supra*) and carbon dioxide, followed by acidification and subsequent esterification with diazomethane, yielded the methyl ester **15** in 76% yield (Scheme 2). The MOM protecting groups were removed using PPTS in methanol at reflux in 88% yield. Subsequent reduction of the ester **16** with lithium aluminum hydride gave the benzyl alcohol **1** in 51% yield.

The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported.[20,21] Treatment of the

bisMOM protected 4-hydroxylalkyl estradiols (**13,14,17**) with phthalimide under Mitsunobu conditions using triphenylphosphine (PPh_3) and diethyl azodicarboxylate (DEAD) yielded derivatives **18-20** in 70-80% yield (Scheme 3). Subsequent hydrazinolysis in refluxing ethanol gave the bisMOM protected aminoestradiols which, upon treatment with methanolic HCl, gave the desired 4-aminoalkylestradiols **4-6** in good yields.

Biology

The affinities of the synthetic 4-hydroxyestradiol analogs for the estrogen receptor were assessed in whole cell estrogen receptor binding assays using MCF-7 human mammary cancer cells.[18] The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations.[14] In addition, the cellular uptake and stability of analogs in the whole cell assay can be assessed. The EC_{50} value for estradiol binding to the estrogen receptor in these whole cell assays was found to be 0.180 nM (Table 1). The synthetic hydroxyestrogen analog with the highest estrogen receptor affinity was 4-hydroxymethylestradiol **1**, exhibiting an EC_{50} value of 364 nM. Overall, the 4-substituted estradiol homologs exhibited significantly weaker affinity for the estrogen receptor than estradiol, with relative binding affinities (RBA; estradiol = 100) ranging from 0.49 for compound **1** to 0.05 for compound **3** (Table 1, Fig. 1).

The relative estrogenic activities of the 4-hydroxyestradiol analogs were evaluated by examining the abilities of the synthetic compounds to induce estrogen-dependent gene expression in human breast cancer cells. In human MCF-7 mammary carcinoma cells, the induction of transcription of the pS2 gene is a primary response to estrogen.[22] The induction of pS2 mRNA expression by estradiol, 4-hydroxyestradiol, and 4-hydroxyalkylestrogen analogs **1** - **3** was determined by RNA dot blot analysis.[21] The EC_{50} value for estradiol induction of pS2 mRNA was found to be 0.030 nM. The estradiol homologs exhibited significantly weaker

activity than estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) ranging from 0.257 for compound **1** to 0.001 for compound **3** (Table 2, Fig. 2).

The effects of 4-hydroxymethylestradiol **1** on the growth of hormone-dependent MCF-7 breast cancer cells was investigated since the compound exhibited the highest estrogen receptor affinity of the 4-hydroxylalkyl analogs synthesized. This mitogenic activity was determined by measuring [^{3}H]-thymidine incorporation at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 4-Hydroxymethylestradiol did not affect cellular DNA synthesis in these breast cancer cell lines, whereas estradiol at a concentration of 1 nM significantly increased MCF-7 cell growth (Fig. 3).

CONCLUSIONS

The Stille cross-coupling and the carboxymethylation reaction reported here represent two efficient, previously unexplored synthetic routes for the functionalization of the 4-position of estradiol. The synthesis of the 4-hydroxyalkyl estrogens was accomplished by oxidative hydroboration of 4-alkenyl estradiols, which were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenyl stannane. The 4-aminoalkyl estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions.

The substituted estradiols were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. The 4-hydroxyalkylestradiols had significantly lower affinity for the estrogen receptor when compared with the endogenous ligand, estradiol. 4-Hydroxymethylestradiol (**1**) exhibited the highest affinity of the synthetic compounds, with an apparent EC_{50} value of 364 nM, and it exhibited a similar affinity as the endogenous metabolite, 4-hydroxyestradiol, in the whole cell

assays. On the other hand, the 4-aminoalkylestradiols (**4** - **6**) exhibited either extremely weak or no affinity for the estrogen receptor.

Estradiol acts through the nuclear estrogen receptor to induce the transcription of a variety of hormone-responsive genes in target tissues, and induction of pS2 gene transcription is a primary response to estrogen observed in human MCF-7 mammary carcinoma cells.[22] The 4-hydroxyalkylestradiols had significantly decreased efficacy for the induction of pS2 mRNA levels in MCF-7 cells when compared with the endogenous ligand, estradiol. Again, 4-hydroxymethylestradiol (**1**) was the most potent among the synthetic compounds, with an apparent EC_{50} value of 11.7 nM. This synthetic compound was more effective than the endogenous metabolite, 4-hydroxyestradiol, which exhibited an apparent EC_{50} value of 65.4 nM.

Thus, the 4-hydroxyalkyl estradiols **1** - **3** exhibited both significantly weaker estrogen receptor affinities and abilities to induce pS2 gene expression in MCF-7 cell cultures. These results are consistent with the established structure-activity relationships of estrogens and the limitations of A-ring substitutions on the estrogen molecule in producing estrogen receptor-mediated responses. On the other hand, 4-hydroxymethylestradiol (**1**) exhibited similar estrogen receptor affinity and similar induction of pS2 gene transcription as the catechol estrogen, 4-hydroxyestradiol. This catechol estradiol has been implicated as a possible causative agent in estrogen-induced tumorigenesis; however, *in vitro* and *in vivo* investigations with 4-hydroxyestradiol are difficult due to its chemical and biochemical instability. Thus, 4-hydroxymethylestradiol (**1**) may be viewed as a chemically stable catechol estrogen homolog and may therefore prove useful in examination of the role of catechol estrogens in normal physiology and in pathological states, such as estrogen-induced tumorigenesis.

EXPERIMENTAL METHODS

Synthesis:

General Information.

Estradiol was purchased from Steraloids (Wilton, NH) . All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. Amines were stirred over CaH_2, distilled and then stored over KOH pellets. Silica gel TLC plates (60 F_{254}) were purchased from Analtech Inc (Newark, NE) and visualized with a UV lamp and/or 5% ethanolic phosphomolybdic acid followed by charring. All intermediates were purified by flash column chromatography on silica gel (Merck Kieselgel 60) using the indicated mixtures of hexanes and ethyl acetate. Melting points were determined in open capillaries on a Thomas Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Laser Precision Analytical RFX-40 FTIR Spectrometer in the phase indicated. ^1H NMR and ^{13}C NMR were recorded on an IBM AF/250 spectrometer at 250 and 67.5 MHz respectively in $CDCl_3$ solutions unless otherwise indicated using the residual protiosolvent signal as internal reference. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center on either a VG 70-2505, a Nicolet FTMS-200 or a Finnigan MAT-900 mass spectrometer. Elemental Analysis were performed by Oneida Research Services, Inc (Whitesboro, NY).

4-Bromoestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (10). MOMCl (5.7 mL, 75 mmol) was added dropwise to a cold (0 °C) solution of 4-bromoestradiol (5.26 g, 15.0 mmol) and di-isopropyl ethylamine (21.3 mL, 89.3 mmol) in THF (125 mL). On completion of the addition, the reaction mixture was allowed to warm up to room temperature, stirred for 1 h at the same temperature, then heated at reflux overnight. The mixture was allowed to cool, then sat. NH_4Cl solution (100 mL) was added. The mixture was extracted with EtOAc (4x100 mL), the combined organic solutions were washed with sat.

aqueous brine (100 mL), dried ($MgSO_4$) and concentrated. The crude product was purified by flash column chromatography (SiO_2, hexane/ethyl acetate, 9:1) to afford a pale yellow solid, which was recrystallized from hexane to give 4.78 g (72%) of the desired compound as a colorless solid: mp 88-89 °C (Lit. 97-98 °C); IR (KBr, cm^{-1}) 2925-2785, 1597, 1578, 1473, 1452, 1444, 1402, 1385, 1306, 1261, 1234, 1224, 1205, 1176, 1155, 1122, 1107, 1088, 980, 914, 897, 856; ^1H NMR 7.20 (1H, d, J = 8.6 Hz), 6.95 (1H, d, J = 8.6 Hz), 5.22 (2H, s), 4.69 (2H, ABq, J = 6.6 Hz, $\Delta\nu$ = 3.5 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.51 (3H, s), 3.37 (3H,s), 2.98 (1H, dd, J = 5.4, 17.9 Hz), 2.76-2.65 (1H, m), 2.31-2.11 (2H, m), 0.79 (3H, s); ^{13}C NMR 151.8, 137.8, 136.5, 124.8, 116.4, 113.5, 96.1, 95.4, 86.7, 56.2, 55.1, 50.1, 44.3, 43.0, 37.9, 37.3, 31.3, 28.2, 27.4, 26.6, 23.1, 11.7; MS *m/z* (M^+) calcd 440.1341, obsd 440.1388.

4-Ethenylestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (11). A solution of **11** (440 mg , 1.0 mmol), vinyl tributyltin (0.62 g, 2.0 mmol) and $Pd(PPh_3)_4$ (67 mg, 0.06 mmol) in DMF (15 mL) was deoxygenated by bubbling argon through it for 15 mins. The solution was heated at reflux overnight, cooled to room temperature and diluted with ether (50 mL), washed with 5% NH_4OH (15 mL), water (4x20 mL), brine (3x20 mL), dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (SiO_2, 4:1 hexane/ethyl acetate) to yield 344 mg (90%) of the title compound as a colorless oil, which solidified on standing to a colorless waxy solid: mp 55 °C; IR (KBr, cm^{-1}) 2930, 2888, 2847, 2023, 1698, 1586, 1476, 1444, 1158, 1112, 1055, 1045, 927; ^1H NMR 7.16 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.9 Hz), 6.40 (1H, dd, J = 11.7, 17.9 Hz), 5.55 (1H, dd. J = 2.3, 17.9 Hz), 5.52 (1H, dd, J = 2.3, 11.7 Hz), 5.12 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.36 (3H, s), 2.91-2.65 (2H, m), 0.74 (3H.s); ^{13}C NMR 152.9, 136.2, 134.4, 131.3, 127.1, 125.0, 119.7, 112.7, 96.1, 95.0, 86.7, 56.0, 55.1, 10.3, 44.5, 43.0, 38.0, 37.5, 28.23, 28.20, 27.4, 26.6, 23.1, 11.7; MS *m/z* (M^+) calcd 386.2457, obsd 386.2443. Anal. ($C_{24}H_{34}O_4$) C,H.

4-(2'-Propenyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (12). A solution of **11** (1.50 g, 3.41 mmol), $Pd(PPh_3)_4$ (250 mg, 0.22 mmol) and allyl tri-n-butyl stannane (2.28 g, 6.90 mmol) in DMF (50 mL) was deoxygenated by bubbling argon through it for 15 min, then the solution was heated at reflux overnight. After cooling, the solution was decanted off from the palladium, and the residual precipitated palladium was washed with ethyl acetate. The reaction solution was diluted with ethyl acetate, then washed with water (3x50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 10:1) to give 1.28 g (94%) of the desired allyl compound **14** as a colorless oil: IR (Neat, cm^{-1}) 2931, 2850, 2825, 1637, 1481, 1446, 1254, 1225, 1205, 1190, 1151, 1134, 1105, 1082, 1055, 1028, 1007, 918; ^1H NMR 7.15 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.97-5.82 (1H, m), 5.17 (2H, s), 4.97 (1H, s), 4.92 (1H, dd, J = 1.5, 7.1 Hz), 4.65 (2H, ABq, J = 6.7, $\Delta\upsilon$ = 2.9 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.42 (2H, t, J = 6.6 Hz), 3.37 (3H, s), 2.91-2.66 (2H, m), 0.79 (3H, s); ^{13}C NMR 153.1, 136.5, 136.3, 134.3, 126.7, 124.1, 114.4, 111.7, 96.1, 94.8, 86.7, 55.9, 55.1, 50.3, 44.4, 43.0, 38.0, 37.5, 30.2, 28.2, 27.4, 26.7, 26.6, 23.1, 11.7; MS *m/z* (M^+) obsd 400.2613, calcd 400.2618. Anal. ($C_{25}H_{36}O_4$) C, H.

4-(2'-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (13). A solution of 1M BH_3•THF (3.00 mL, 3.00 mmol) was added dropwise to a solution of **12** (286 mg, 0.75 mmol) in THF (6 mL) at 0 °C. On completion of the addition the cooling bath was removed and the mixture stirred for 1 h; 1M NaOH (3 mL) was added cautiously and, after the addition of 30% H_2O_2 (3 mL), the mixture was heated at reflux for 1 h. The mixture was allowed to cool, then ethyl acetate (75 mL) was added and the organic solution was separated from the aqueous layer. The organics were washed with water (25 mL), brine (25 mL), dried ($MgSO_4$) and concentrated. The residue was purified by MPLC (SiO_2, hexane/ethyl acetate, 2:1) to give 37 mg (12%) of a diastereomeric mixture of partially

deprotected secondary alcohols, 92 mg (30%) of a mixture of two diastereomeric secondary alcohols and 119 mg (39%) of the desired primary alcohol as a colorless oil, which crystallized on standing: mp 81-82 °C; IR (KBr, cm^{-1}) 3496, 3311, 2931, 2870, 2844, 2821, 1595, 1581, 1481, 1404, 1385, 1309, 1255, 1227, 1205, 1190, 1149, 1111, 1096, 1068, 1053, 1009, 912, 814; ^1H NMR 7.12 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, ABq, J = 6.6, $\Delta\upsilon$ = 3.3 Hz), 3.78 (2H, t, J = 7.0 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.46 (3H, s), 3.36 (3H, s), 2.97 (2H, t, J = 6.7 Hz), 2.96-2.88 (1H, m), 2.82-2.70 (1H, m), 0.79 (3H, s); ^{13}C NMR 153.6, 136.7, 134.6, 125.4, 124.4, 111.5, 96.1, 94.7, 86.7, 62.2, 56.0, 55.1, 50.3, 44.4, 43.0, 37.9, 37.4, 29.5, 28.2, 27.4, 27.1, 26.6, 23.1, 11.7; MS *m/z* (M^+) calcd 404.2563, obsd 404.2574. Anal. ($C_{24}H_{36}O_5$) C, H.

4-(3'-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (14). A solution of 1M BH_3•THF (12.4 mL, 12.4 mmol) was added dropwise to a solution of **14** (1.23 g, 3.08 mmol) in THF (25 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 1 h. 1M NaOH (25 mL) was added cautiously, then 30% H_2O_2 (25mL) was added and the resulting mixture heated at reflux for 1 h. The aqueous reaction mixture was extracted with ethyl acetate (3x100 mL) and the organic layer was washed with water (100 mL), brine (100 mL), dried ($MgSO_4$) and concentrated. Column chromatography (SiO_2, hexane/ethyl acetate, 2:1) of the residue gave 1.06 g (82%) of the desired alcohol as a colorless oil; IR (Neat, cm^{-1}) 3442, 2927, 1479, 1254, 1205, 1151, 1105, 1053, 1024, 920; ^1H NMR 7.13 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, ABq, J = 7.0, $\Delta\nu$ = 0 Hz), 3.67-3.37 (3H, m), 3.48 (3H, s), 3.37 (3H, m), 0.79 (3H, s), ^{13}C NMR 153.3, 136.2, 134.7, 128.6, 123.8, 111.5, 96.1, 95.0, 86.7, 62.4, 86.1, 55.1, 50.3, 44.4, 43.0, 38.0, 37.4, 32.0, 28.2, 27.4, 26.5, 23.1, 21.8, 11.7; MS *m/z* (M^+) calcd 418.2719, obsd 418.2712. Anal. ($C_{25}H_{38}O_5$) C, H.

4-(2'-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol (2). A solution of **13** (80 mg, 0.20 mmol) and pyridinium *p*-toluenesufonate (0.50 g, 2.00 mmol) in methanol (5 mL) were heated at reflux for 24 h. After cooling to room temperature, ethyl acetate (50 mL) was added and then the solution was washed with water (2x50 mL) and brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was recrystallized from methanol and water to afford 50 mg (80%) of the alcohol: mp 229-230 °C; IR (KBr, cm^{-1}) 3338, 2966-2860, 1591, 1481, 1469, 1444, 1425, 1377, 1358, 1340, 1277, 1200, 1180, 1134. 1072, 1057, 1039, 1011, 818, 810; 1H NMR (DMSO) 8.89 (1H, s), 6.92 (1H, d, *J* = 8.48 Hz), 6.56 (1H, d, J = 8.4 Hz), 4.62 (1H, br), 4.47 (1H, d, J = 4.8 Hz), 3.54-3.41 (1H, m), 2.84-2.59 (4H, m), 0.63 (3H, s); 13C NMR 151.9, 135.5, 130.7, 123.2, 122.7, 112.1, 79.9, 59.9, 49.5, 43.7, 42.5, 37.8, 36.5, 29.8, 29.4, 27.0, 26.1, 22.6, 11.0; (M^+) calcd 316.2038, obsd 316.2032. Anal. ($C_{20}H_{28}O_3 \bullet 0.5H_2O$) C, H.

4-(3'-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol (3). A solution of the alcohol **14** (173 mg, 0.41 mmol) and pyridinium p-toluenesulfonate (0.50 g, 2.0 mmol) in methanol (5 mL) was heated at reflux for 24h. After cooling, the reaction mixture was diluted with ethyl acetate (50 ml), then it was washed with water (3x25 mL), brine (25 mL), dried and concentrated. The residue was recrystallized from methanol/water to give 61 mg (61%) of the desired alcohol as a colorless solid: mp 240-242 °C; IR (KBr, cm^{-1}) 3392, 3249, 2971, 2916, 2864, 1591, 1491, 1471, 1446, 1425, 1379, 1362, 1280, 1080, 1059, 1034, 1003, 814, 808; ^{1}H NMR (DMSO) 10.28 (1H, s), 6.90 (1H, d, J = 8.5 Hz), 6.56 (1H, d, 8.5 Hz), 4.47 (1H, d, J = 4.8 Hz), 4.41 (1H, t, J = 5.2 Hz), 3.54-3.36 (2H, m), 2.80-2.45 (5H, m), 2.22-2.12 (1H, m), 2.09-2.00 (1H, m), 1.84-1.80 (3H, m), 1.59-1.43 (3H, m), 1.39-1.04 (7H, m), 0.54 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 125.9, 122.8, 112.1, 79.9, 60.9, 49.6, 43.8, 42.6, 37.9, 36.5, 31.8, 27.0, 26.2, 25.9, 22.6, 21.8, 11.1; MS m/z (M+) calcd 330.2195, obsd 330.2192. Anal. ($C_{21}H_{30}O_3 \bullet 0.25H_2O$) C, H.

4-Carboxy-3,17β-Bis(methoxymethoxy)estra-1,3,5(10)-triene 3,17β-diol Methyl Ester (15) n-BuLi (3.5 mL, 5.65 mmol) was added dropwise to a solution of **10** (1.15 g, 2.61 mmol) in THF (50 mL) at -78 °C. After stirring for 1h at this temperature, several pieces of dry-ice were added and then the cooling bath was removed. After warming to room temperature, the reaction mixture was diluted with ether (50 mL) and then extracted with 5% KOH (5x50 mL). The combined basic extracts were acidified to pH 5 with concentrated HCl, then extracted with ether (6x50 mL). The combined ethereal extracts were washed with brine (50 mL), dried ($MgSO_4$) and concentrated to give the crude acid. The acid was suspended in ether (20 mL) and an ethereal solution of diazomethane was added to it at 0 °C. After 30 min., sufficient acetic acid was added to the reaction mixture to discharge the yellow coloration. The reaction mixture was washed with $NaHCO_3$ (2x50 mL), brine (50 mL), dried ($MgSO_4$), concentrated and purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to give 0.82 g (76%) of the desired product as a colorless oil, which crystallized after a few days standing at room temperature: mp 66-68 °C; IR (KBr, cm^{-1}) 2924, 2862, 1728, 1585, 1481, 1440, 1384, 1273, 1257, 1248, 1155, 1126, 1103, 1061, 1049, 1031, 793; ^1H NMR 7.25 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.13 (2H, s), 4.64 (2H, ABq, J = 7.0, $\Delta\nu$ = 0 Hz), 3.88 (3H, s), 3.59 (1H, t, J = 8.4 Hz), 3.44 (3H, s), 3.36 (3H, s), 2.79-2.73 (2H, m), 2.29-1.99 (4H, m), 1.95-1.83 (1H, m), 0.79 (3H, s); ^{13}C NMR 168.9, 151.6, 134.7, 134.6, 127.4, 124.8, 112.6, 96.2, 95.0, 86.7, 56.0, 55.1, 51.9, 50.1, 44.1, 43.0, 38.1, 37.3, 28.2, 26.8, 26.6, 26.4, 23.1, 11.7; MS *m/z* (M^+) calcd 418.2355 found 418.2337. Anal. ($C_{24}H_{34}O_6$) C, H.

4-Carboxy-3,17β-estra-1,3,5(10)-triene 3,17β-diol Methyl Ester (16). A solution of the ester **15** (0.44 g, 1.10 mmol) and pyridinium *p*-toluenesulfonate (2.77 g, 11.0 mmol) in MeOH (10 mL) was heated to reflux for 36 h. After cooling and addition of EtOAc (100 mL), the organic solution was washed with water (2x50 mL), brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was filtered through a short pad of silica gel (ethyl acetate/hexane 1:2) to give 0.30 g (83%) of **16** as a colorless oil, which crystallized on

standing: mp 135-136 °C (MeOH/H_2O); IR (KBr, cm^{-1}) 3435, 2920, 2866, 1718, 1591, 1446, 1427, 1383, 1361, 1344, 1288, 1267, 1230, 1217, 1190, 1171, 1136, 1122, 1059, 1039, 1011, 960; ^1H NMR 10.72 (1H, s), 7.38 (1H, d, J = 8.8 Hz), 6.80 (1H, d, J = 8.8 Hz), 3.93 (3H, s), 3.72 (1H, t, J = 8.4 Hz), 3.10-3.04 (2H, m), 2.29-2.06 (3H, m), 1.97-1.84 (2H, m), 0.77 (3H, s); ^{13}C NMR 172.0, 160.0, 139.3, 132.4, 132.1, 115.2, 112.7, 81.8, 51.9, 501, 44.7, 43.3, 37.9, 36.9, 30.7, 29.8, 27.3, 26.9, 23.0, 11.1; MS m/z (M^+) calcd 330.1831 found 330.1835. Anal. ($C_{20}H_{26}O_4 \bullet 0.5H_2O$) C, H.

4-Hydroxymethylestra-1,3,5(10)-triene 3,17β-diol (1). $LiAlH_4$ (70 mg, 1.89 mmol) was added portionwise to a solution of the ester **16** (51 mg, 0.15 mmol) in THF (5 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 4 h. When the reaction was complete, water (0.07 mL), 15% NaOH (0.07 mL) and water (0.21 mL) were added. Once a granular precipitate had formed, it was removed by suction filtration through Celite, washed with MeOH, and concentrated to yield 23 mg (51%) of the desired alcohol as a colorless solid; mp > 270 °C; IR (KBr, cm^{-1}) 3375, 3240, 2960, 2931, 2920, 2866, 2850, 1591, 1479, 1448, 1429, 1383, 1352, 1286, 1252, 1078, 1065, 1009, 820 ^1H NMR 9.01 (1H, s), 6.99 (1H, d, J = 8.5 Hz), 6.58 (1H, d, J = 8.5 Hz), 4.53 (1H, m), 4.48 (2H, ABq, J = 4.8, $\Delta\nu$ = 7.7 Hz), 3.55-3.50 (1H, m), 2.98-2.80 (1H, m), 2.78-2.63 (1H, m), 2.30-2.05 (1H, m), 2.03-1.86 (1H, m), 1.85-1.63 (3H, m), 0.67 (3H, s); ^{13}C NMR 153.1, 136.3, 130.7, 124.7, 124.4, 112.7, 79.9, 54.7, 49.5, 43.7, 42.6, 37.9, 36.5, 29.8, 26.8, 26.2, 25.4, 22.6, 11.0; MS m/z (M^+) calcd 302.1875, obsd 302. 1883. Anal. ($C_{19}H_{26}O_3 \bullet 0.25H_2O$) C, H.

4-Hydroxymethyl-3,17β-bis(methoxymethoxy)estra-1,3,5(10)triene (17). $LiAlH_4$ (340 mg, 9.19 mmol) was added portionwise to a solution of **15** (0.50 g, 1.20 mmol) in THF (30 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature over 1 h, then stirred for 4h. Water (0.34 mL), 15% NaOH (0.34 mL) and water (1.00 mL) were added

successively, then the resulting grannular precipitate was removed by filtration through a pad of Celite and $MgSO_4$ (1:1). After concentration of the filtrate it was chromatographed to give 0.43 g (90%) of the desired alcohol as a colorless oil, which slowly crystallized: mp 80-81 °C; IR (KBr, cm^{-1}) 3479, 2964-2814, 1597, 1583, 1481, 1441, 1400, 1385, 1255, 1242, 1228, 1153, 1107, 1092, 1063, 1045, 1031, 1005, 995, 955, 903; ^1H NMR 7.23 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.7 Hz), 5.19 (2H, ABq, J = 6.7, $\Delta\nu$ = 3.4 Hz), 4.74 (2H, m), 4.64 (2H, ABq, J = 6.6, $\Delta\nu$ = 3.4 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.48 (3H, s), 3.36 (3H, s), 3.10-2.92 (1H, m), 2.90-2.81 (1H, m), 2.33-1.91 (6H, m), 1.76-1.11 (9H, m), 0.79 (3H, m); ^{13}C NMR 153.9, 136.8, 135.1, 128.0, 126.2, 112.6, 96.1, 95.5, 86.7, 56.7, 56.3, 55.1, 50.2, 44.4, 43.0, 37.9, 37.4, 28.2, 27.2, 26.6, 26.5, 23.1, 11.7; MS *m/z* (M^+) calcd 390.2397 obsd 390.2391. Anal. ($C_{23}H_{34}O_5$) C, H.

Phthalimides 23-24: General Procedure: DEAD (0.42 mL, 2.40 mmol) was added dropwise to a solution of the alcohol **13, 14,** or **17** (0.80 mmol), phthalimide (0.35 g, 2.40 mmol) and triphenylphosphine (0.63 g, 2.40 mmol) in THF (10 mL) at room temperature, then stirred overnight. The solvent was removed *in vacuo* then the residue was dissolved in EtOAc (100 mL) and washed with 5% aqueous KOH (4x50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to afford the substituted phthalimides **18-20** as colorless or pale yellow oils, which solidified on standing:

4-(Phthalimidoylmethyl)-3,17β-bis(methoxymethoxy)estra-1,3,5(10)triene (18). 79%: mp 104-106 °C; IR (KBr, cm^{-1}) 2951, 2925, 2868, 1711, 1479, 1396, 1385, 1348, 1255, 1151, 1113, 1092, 1055, 1028, 1009, 993, 962, 949, 918, 723; ^1H NMR 7.79-7.74 (2H, m), 7.69-7.64 (2H, m), 7.20 (1H, d, J = 8.7 Hz), 6.90 (1H, d, J = 8.7 Hz), 5.12 (2H, s), 4.90 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.36 (3H, s), 3.31 (3H, s), 3.20-3.13 (1H, m), 2.97-2.86 (1H, m), 2.29-1.94 (3H, m), 0.78 (3H, s); ^{13}C NMR 167.9, 137.6, 134.2, 133.7, 132.3, 126.0, 123.0, 122.0,

111.4, 96.1, 94.6, 86.7, 55.8, 55.1, 50.3, 44.4, 43.0, 37.8, 37.4, 34.3, 28.2, 27.4, 26.9, 26.5, 23.1, 11.7; MS *m/z* (M^+) calcd 519.2621, obsd 519.2634. Anal. ($C_{32}H_{39}NO_6$) C, H, N.

4-(Phthalimidoylethyl)-3,17β-bis(methoxymethoxy)estra-1,3,5(10)triene (19). 87%: mp 117-120°C; IR (KBr, cm^{-1}) 2930, 2880, 1772, 1716, 1505, 1430, 1393, 1360, 1152, 1119, 1109, 1070, 1052, 998, 900, 720; ^1H NMR 7.83-7.67 (4H, m), 7.15 (1H, d, J = 8.68 Hz), 6.90 (1H, d, J = 8.67 Hz), 5.16 (2H, s), 4.64 (2H, ABq, J = 6.8, $\Delta\nu$ = 2.9 Hz), 3.81 (1H, t, J = 8.2 Hz), 3.48 (3H, s), 3.35 (3H, s), 3.03-2.75 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.1, 153.6, 136.6, 134.4, 133.7, 132.3, 124.9, 124.6, 123.0, 111.2, 96.0, 94.5, 86.6, 56.0, 50.1, 44.3, 42.9, 37.8, 37.3, 36.8, 28.1, 27.3, 26.7, 26.5, 25.3, 23.0, 11.7; MS *m/z* (M^+) calcd 533.2767. obsd. 533.2772 Anal. ($C_{31}H_{37}NO_6$) C, H, N.

4-(Phthalimidoylpropyl)-3,17β-bis(methoxymethoxy)estra-1,3,5(10)triene (25). 93%: mp 134-135°C; IR (KBr, cm^{-1}) 2955, 2935, 2894, 2786, 1777, 1726, 1485, 1478, 1445, 1394, 1363, 1153, 1122, 1086, 1055, 1040, 922, 720; ^1H NMR 7.85-7.80 (2H, m), 7.73-7.67 (2H, m), 7.13 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 5.09 (2H, s), 4.64 (2H, ABq, J=6.9, $\Delta\nu$=1.7 Hz), 3.78 (2H, t, J=7.2 Hz), 3.59 (1H, t, J=8.3 Hz), 3.39 (3H, s), 3.36 (3H, s), 2.88-2.64 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.3, 153.0, 135.7, 134.1, 133.7, 132.2, 128.2, 123.7, 123.0, 111.2, 96.0, 94.4, 86.7, 55.8, 55.0, 50.2, 44.3, 42.9, 38.4, 37.8, 37.4, 28.1, 27.4, 26.6, 26.5, 23.3, 23.0, 11.6; MS *m/z* (M^+) calcd 547.1693, obsd 547.2908. Anal. ($C_{33}H_{41}NO_6$) C, H, N.

Amines 4-6: General Procedure: A solution of the phthalimides **18-20** (0.46 mmol) and hydrazine (1 mL) in ethanol (10 mL) were heated at reflux for 1h. After cooling, ethyl acetate (50 mL) was added and then the mixture was washed with 5% KOH solution (3x25 mL), brine (25 mL), dried ($MgSO_4$) and concentrated. The residue was dissolved in methanol (5 mL), cooled to 0 °C and then HCl was bubbled through it for 15 min. The cooling bath was removed and then stirring was continued for 3h. The reaction mixture was concentrated, then redissolved

in methanol and the resulting green solution was decolorized with charcoal. After filtration and concentration the residue was taken up in the minimum amount of methanol and the product was precipitated out by the addition of ether, affording the amine salts as colorless or yellow solids.

4-Aminomethylestra-1,3,5(10)-triene 3,17β-diol (4). 90%: mp > 270 °C; IR (KBr, cm^{-1}) 3444-2868, 1620, 1591, 1509, 1491, 1473, 1450, 1379, 1352, 1323, 1284, 1261, 1219, 1201, 1188, 1080, 1057, 1007, 945, 814; ^1H NMR (DMSO) 8.42 (4H, brs), 7.15 (1H, d, J = 8.6 Hz), 6.75 (1H, d, J = 8.6 Hz), 4.48 (1H, brs), 3.89 (1H, s), 3.52 (1H, *t*, J = 8.2 Hz), 2.94-2.70 (2H, m), 2.30-2.22 (1H, m), 1.95-1.70 (3H, m), 1.65-1.49), 0.64 (3H, s); ^{13}C NMR 152.9, 135.4, 130.1, 125.5, 116.8, 111.5, 78.8, 48.3, 42.6, 41.5, 36.7, 35.4, 32.9, 28.8, 25.6, 25.2, 24.8, 21.6, 10.0; MS *m/z* (M^+-HCl) calcd 301.2037, obsd 301.2042. Anal. ($C_{19}H_{28}NO_2Cl \bullet H_2O$) C, H, N.

4-Aminoethylestra-1,3,5(10)-triene 3,17β-diol (5). 87 %: mp > 270 °C; IR (KBr, cm^{-1}) 3355, 3299, 3059, 2865, 1589, 1471, 1447, 1383, 1362, 1281, 1270, 1142, 1086, 1066, 1020, 943, 809; ^1H NMR (DMSO) 8.5 (4H, brs), 6.92 (1H, d, J = 8.12 Hz), 6.54 (1H, d, J = 8.03 Hz), 4.49 (1H, brs), 3.52 (1H, *t*, J = 8.01 Hz), 2.80-2.55 (4H, m), 0.64 (3H, s); ^{13}C NMR 153.7, 134.9, 131.2, 123.1, 113.0, 79.8, 49.7, 44.9, 42.4, 38.1, 36.2, 29.9, 27.0, 26.3, 26.1, 22.9, 11.3; MS *m/z* (M^+-HCl) calcd 315.2198, obsd 315.2201.

4-Aminopropylestra-1,3,5(10)-triene 3,17β-diol (6). 86%: mp > 270 °C; IR (KBr, cm^{-1}) 3362, 3276, 3056, 3022, 2962, 2925, 2863, 1635, 1589, 1489, 1443, 1280, 1208, 1133, 1058, 813 ; ^1H NMR (DMSO) 9.2 (1H, brs), 7.91 (3H, s), 6.94 (1H, d, J = 8.49 Hz), 6.62 (1H, d, J = 8.38 Hz), 4.50 (1H, d, J = 4.6 Hz), 3.51-3.44 (3H, m), 2.78-2.73 (4H, m), 0.63 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 124.4, 123.2, 112.2, 79.8, 49.5, 43.7, 42.5, 40.5, 37.8, 36.4, 29.8, 26.9, 26.3, 26.1, 25.8, 22.6, 22.0, 11.0; MS *m/z* (M^+-HCl) calcd 329.2355, obsd 329.2354.

Biological Evaluations:

General Information. [2,4,6,7-^{3}H]Estradiol (98.4 Ci/mmol, ^{3}H-E_2) was purchased from Dupont/NEN (Boston, MA) and was used as received. MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and cells were incubated in a humidified CO_2 incubator (Forma model 3052) with 5% CO_2 atmosphere. A modified Eagle's minimum essential medium (MEM) supplemented with essential amino acids (1.5x), vitamins (1.5x), nonessential amino acids (2x) and l-glutamine (1x) was obtained from Gibco BRL (Long Island, NY) and was used for maintaining the cells. The sterilized liquid medium was prepared by the OSU Comprehensive Cancer Center by dissolving the powder in water containing sodium chloride (0.487 g/L), pyruvic acid (0.11 g/L), sodium bicarbonate (1.5 g/L) and the pH adjusted to 6.8. Fetal calf serum was obtained from Gibco BRL. Steroids were removed from heat-inactivated fetal calf serum by two treatments with dextran-coated charcoal at 57°C. Tissue culture flasks and supplies were obtained from Corning Glass Works (Corning, NY). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 693 (Dupont/NEN) as the counting solution. Probes for RNA dot blot analysis (pS2:ATCC 57137; 36B4:ATCC 65917) were obtained as purified plasmids from the American Type Culture Collection and amplified by PCR for use in hybridization. Primers used were synthesized by OLIGOS, ETC (Wilsonville, OR) and were:

For pS2:	sense	5'-ATC CCT GAC TCG GGG TCG CCT TTG-3'
	antisense	5'-CAA TCT GTG TTG TGA GCC GAG GCA CAG-3'
For 36B4:	sense	5'-AAA CTG CTG CCT CAT ATC CG-3'
	antisense	5'-TTT CAG CAA GTG GGA AGG TG-3'

Probes were labelled by random priming with Klenow fragment. Analysis of the RNA dot blots were performed on a Molecular Dynamics PhosphoImager SI.

Whole Cell Estrogen Receptor Studies:[20] MCF-7 cells were maintained in a similar fashion as described above. Cells from 90-100% confluent cultures were harvested by treatment with 0.01% trypsin solution and the washed cell pellet was divided into 9.4 cm^2 wells on a six well plate at 1.5-2x10^5 cells/well in modified MEM (2-3 mL) containing 10% steroid free fetal calf serum and gentamycin (20 mg/mL). After 12-24 h at 37 °C, the culture media was removed and replaced and with serum-free modified MEM media (888 μL) containing insulin (5.0 mg/L), transferrin (5.0 mg/L), glutamine (2 mM) and albumin (2.0 mg/mL). After 48 hours, the media was removed, fresh serum-free modified MEM media added, and the synthetic estrogen **1 - 6** at various concentrations (3x10^{-5} to 1x10^{-5} M, 100 μL) were added and incubated for 10 min at 37 °C. To determine total binding, [^{3}H]-estradiol (3.0 nM, 1.0 μCi) was added and the plates were then incubated for 1 h at 37°C. The cells were washed twice with PBS at 4 °C then 95% ethanol (1 mL) was added, followed by standing for 30 min. at room temperature. An aliquot (500 μL) of the ethanol solution was added to Formula 963 and counted on a liquid scintillation counter. The blank samples with no cells and nonspecific binding samples, containing 6 μM unlabeled estradiol, were performed in a comparable manner. Specific binding of [^{3}H]-estradiol was calculated by subtracting the nonspecific binding data from total binding data. The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [^{3}H]-estradiol binding and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

pS2 Induction: MCF-7 cells were maintained in a similar fashion as described above. Cells were plated at a concentration of 5.5 x 10^5 cells/25 cm^2 flask. After 2 days of growth, the cells were rinsed with Ca^{++}, Mg^{++} free PBS and placed on defined media for 48 hours. Defined media contained DMEM/F12 media (Gibco BRL) supplemented with human albumin (2.0 mg/ml), transferrin (5.0 mg/L), bovine insulin (5.0 mg/L), and *l*-glutamine (2 mM). After addition of fresh defined media, the cells were dosed with compound (10^{-10} M to 10^{-5} M), 10nM 17β-estradiol (Sigma, St. Louis, MO) or carrier (95% ethanol). Each compound was

tested in triplicate. After 24 hours, total cellular RNA was isolated by an adaptation of the method of Chomczynski and Sacchi.[20] The cells were lysed with a 4M guanidine isothiocyanate solution, and the lysate acidified with 3M sodium acetate, pH 5.2 (1:10 vol). After addition of 3M NaOAc, pH 5.2 (1:10 vol.), RNA was extracted twice using water saturated phenol:chloroform:isoamyl alcohol (60:24:1) at pH 4.0. A final extraction using an equal volume of chloroform:isoamyl alcohol (25:1) was performed. RNA from the resulting aqueous layer was precipitated with an equal volume of isopropanol at -20°C for one hour. The RNA was pelleted at 15,000xg for 30 minutes at 4° C. The resulting pellet was washed 2X with 70% ethanol and 1X with 95% ethanol. Dried pellets were resuspended in 30*ul* of Dnase-, Rnase- free molecular biology grade water (Sigma Chemical Co.). Quantification of RNA in each sample was performed using the absorbance at 260nm.

Dot Blot Analysis: A denaturing solution containing 50% formamide, 7% formaldehyde, and 1X SSPE was added to 15 μg of RNA from each sample. The RNA was denatured at 68°C for 15 minutes. Two volumes of 10X SSPE was added to each sample. The samples were loaded onto a 0.45 μm, positively charged, nylon membrane (Schleicher and Schuell, Keene, NH) using gentle suction through a 96-well dot blot manifold (BioRad, Hercules, CA). Membranes probed for pS2 gene expression were loaded with 10 μg RNA, the remaining 5 μg was loaded onto a membrane probed for the control gene, 36B4. Membranes were baked at 80° C for 1 hour and then incubated for at least 3 hours in a pre-hydridization solution containing 5X SSPE, 5X Denhardts Reagent, 2% SDS, 100 ug/ml salmon sperm DNA and 50% formamide. pS2 and 36B4 cDNA was prepared as described above and used to make ^{32}P-radiolabelled probes using random primers in the RadPrime Kit (Gibco BRL). Probes with specific activity ranging from 5.0×10^5 to 2.0×10^6 cpm/ng were used. Membranes, probed separately for pS2 or 36B4, were incubated for 48 hours or 24 hours, respectively, in hybridization solution containing 5X SSPE, 5X Denhardts reagent, 1% SDS, 100 ug/ml salmon sperm DNA, 10% PEG, and 50% formamide. The membranes were washed in: 0.5X SSPE, 60', at 55°C; 0.1X

SSPE, 60', 60°C; and 0.1X SSPE, 60', 65°C. Phosphor screens were exposed for at least one hour and scanned on the PhosphorImager SI (Molecular Dynamics). Quantification of the signal was performed using ImageQuaNT™ software (Molecular Dynamics). The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal induction of pS2 mRNA and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

Cell Growth Assay: Human mammary carcinoma cell lines were maintained in 75-cm^2 plastic flasks at 37°C in a modified Eagle's MEM (10 ml) containing 10% fetal calf serum and gentamycin (20 mg/l). For determination of the effects of the synthetic 4-hydroxymethylestradiol **1** on cell growth, the mammary carcinoma cells were divided into 9.4 cm^2 wells at approximately 100,000 cells/well in modified MEM (2 ml) containing 10% steroid-free fetal calf serum and gentamycin (20 mg/l). After two days, media was changed to serum-free modified MEM and experiments initiated. Cell number was determined by measuring the DNA content of the cultures using the diphenylamine assay (25). Effects on cell division were measured by the addition of [^{3}H]-thymidine (1 μCi/well), followed by incubation for 2 hours, cell lysis, and determination of [^{3}H]-thymidine incorporation into DNA. To determine dose-dependent effects, varying concentrations of 4-hydroxymethylestradiol (3 nM to 10 μM in 5 μl 95% ethanol) were added and incubated for 4 days. Each experiment was carried out in quadriplicates, and test compounds were evaluated in experiments performed at least three different times. Statistical differences between control and treated groups were determined using the Student's *t* test.

ACKNOWLEDGMENTS

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Table 1.

Estrogen Receptor Affinity of 4-Substituted Estradiol Analogs

steroid	cpd	EC_{50}	Log EC_{50} ± SD	RBA
estradiol		1.80×10^{-10} M	-9.744 ± 0.102	100.00
4-hydroxyestradiol		5.06×10^{-7} M	-6.295 ± 0.092	0.36
4-hydroxymethylestradiol	**1**	3.64×10^{-7} M	-6.438 ± 0.141	0.49
4-hydroxyethylestradiol	**2**	6.20×10^{-7} M	-6.207 ± 0.290	0.29
4-hydroxypropylestradiol	**3**	3.32×10^{-6} M	-5.479 ± 0.116	0.05
4-aminomethylestradiol	**4**	NB		-
4-aminoethylestradiol	**5**	2.50×10^{-6} M	-5.600 ± 0.159	0.07
4-aminopropylestradiol	**6**	NB		-

NB = no measurable binding of steroid at 10^{-5} M concentration

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Table 2.

Induction of pS2 Gene Expression by 4-Substituted Estradiol Analogs

steroid	cpd	EC_{50}	Log EC_{50} ± SD	%Relative Activity
estradiol		3.01×10^{-11} M	-10.520 ± 0.217	100.00
4-hydroxyestradiol		6.54×10^{-8} M	-7.184 ± 0.158	0.046
4-hydroxymethylestradiol	**1**	1.17×10^{-8} M	-7.933 ± 0.288	0.257
4-hydroxyethylestradiol	**2**	1.48×10^{-7} M	-6.829 ± 0.094	0.020
4-hydroxypropylestradiol	**3**	2.95×10^{-6} M	-5.530 ± 0.217	0.001

Figure Legends

Figure 1. Estrogen receptor competitive binding assays for estradiol (■), 4-hydroxy-methylestradiol (●), and 4-hydroxypropylestradiol (□).

Figure 2. Induction of pS2 gene expression by estradiol (●), 4-hydroxyestradiol (▲), 4-hydroxymethylestradiol (□), 4-hydroxyethylestradiol (▽), and 4-hydroxypropylestradiol (◆).

Figure 3. Comparison of mitogenic activities of estradiol (■), 4-hydroxymethylestradiol (■), and vehicle control (□) in MCF-7 human mammary carcinoma cell cultures.

Figure 1

MCF-7 Estrogen Receptor Competitive Binding Assay

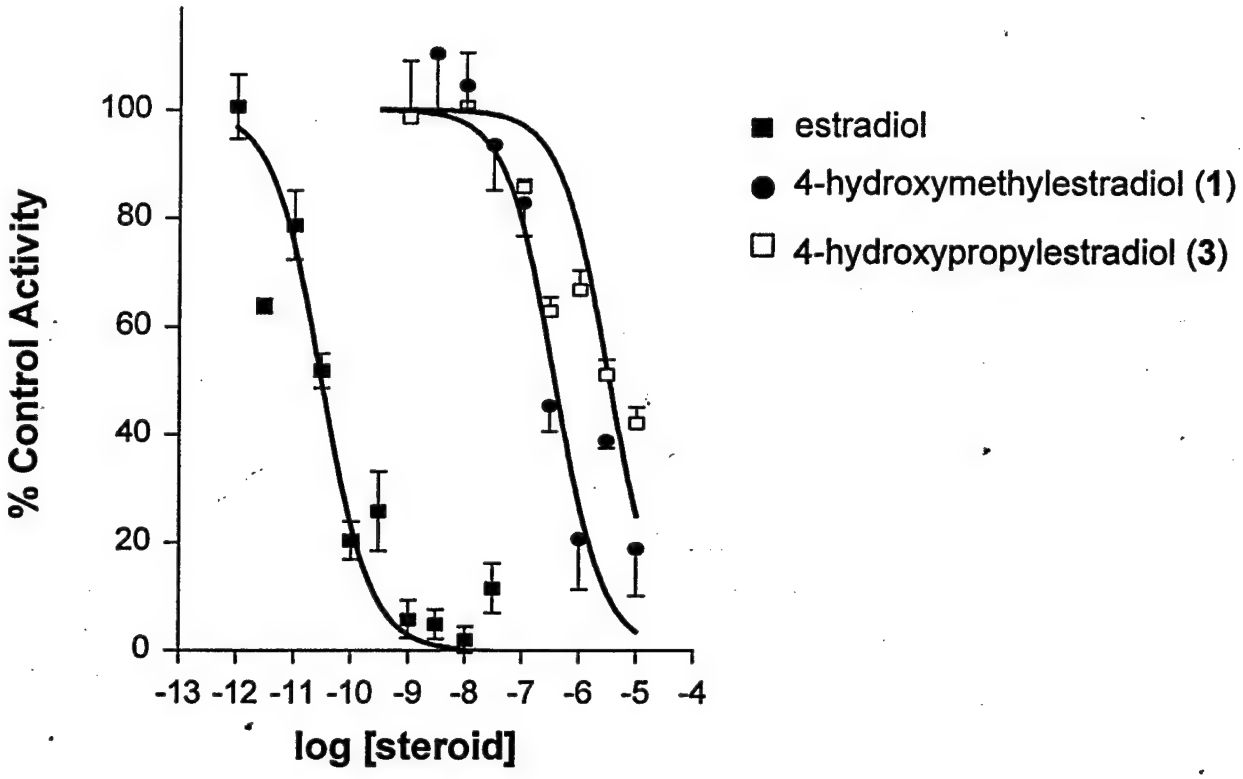


Figure 2

Induction of pS2 Gene Expression

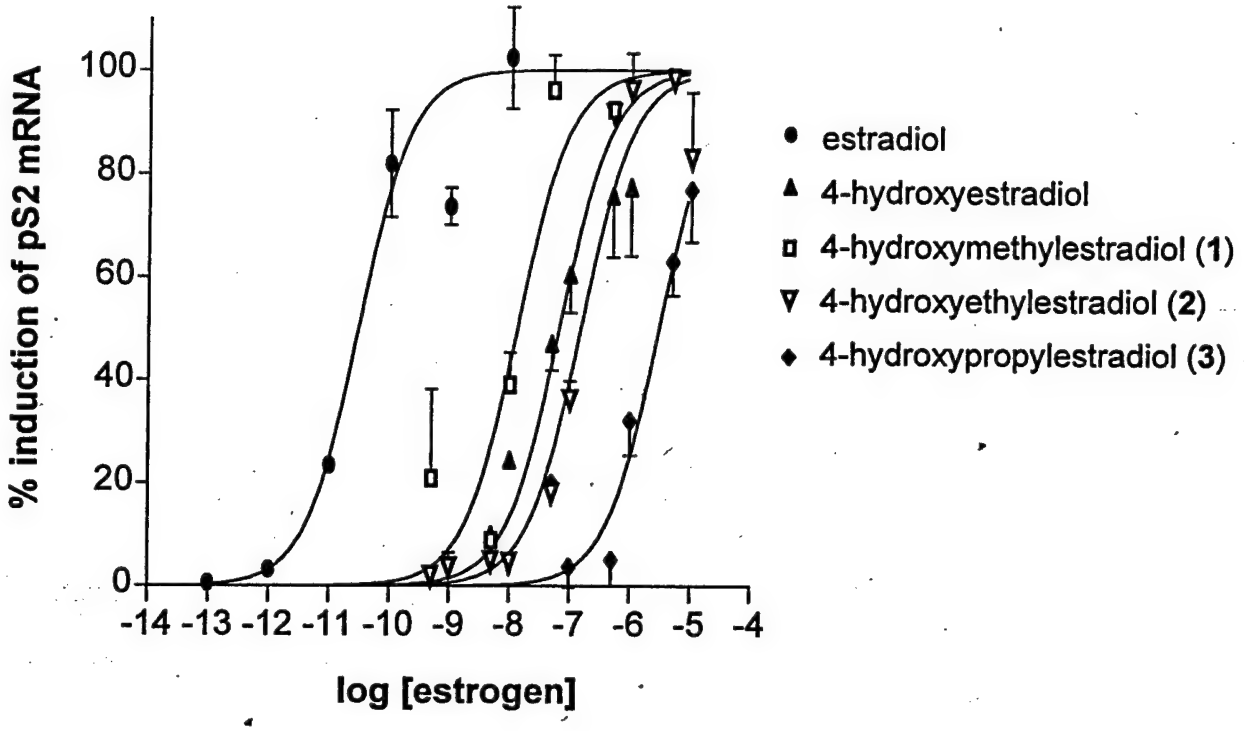
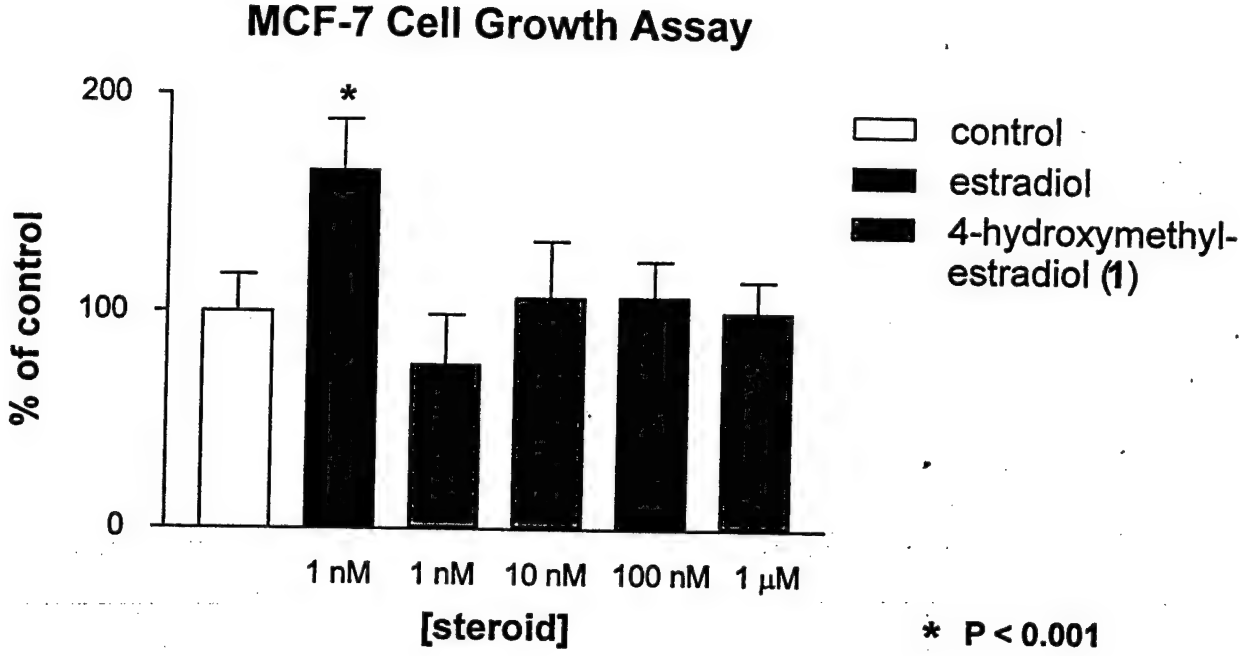
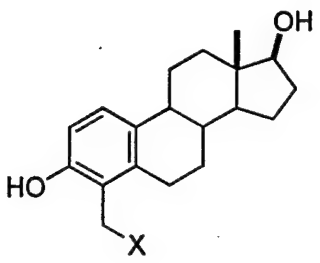


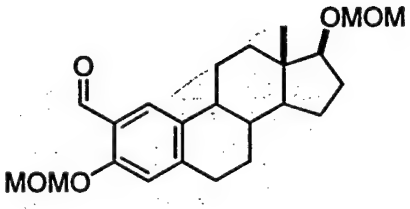
Figure 3


MCF-7 Cell Growth Assay
200
100
0
% of control
*
1 nM
1 nM
10 nM
100 nM
1 μM
[steroid]
control
estradiol
4-hydroxymethyl-estradiol (1)
* P < 0.001

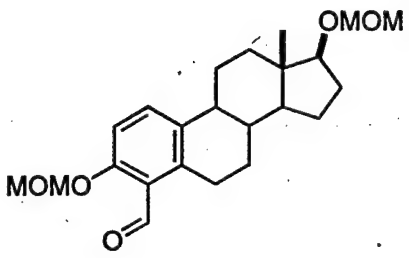
Synthetic Targets and Intermediates



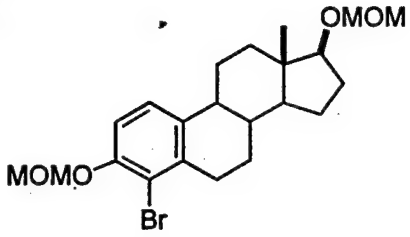
1: X=OH
2: X=CH_2OH
3: X=CH_2CH_2OH
4: X=NH_2
5: X=CH_2NH_2
6: X=$CH_2CH_2NH_2$



7

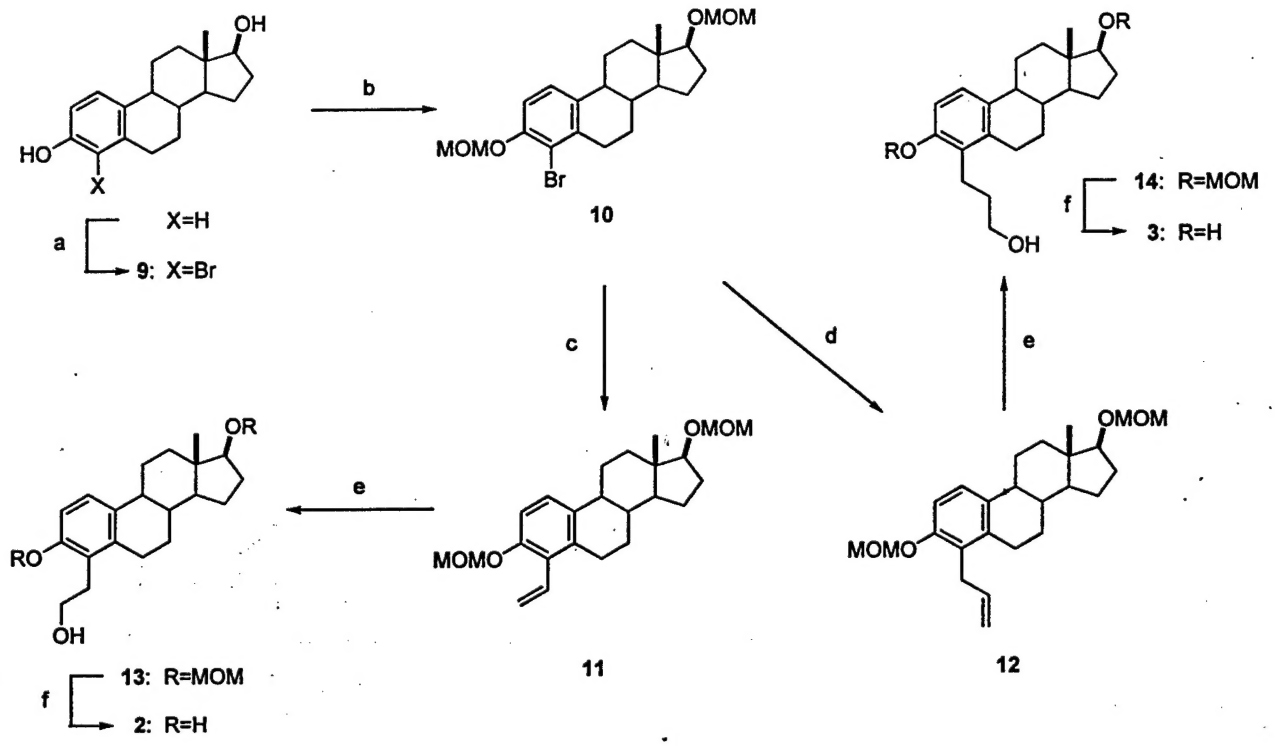


8



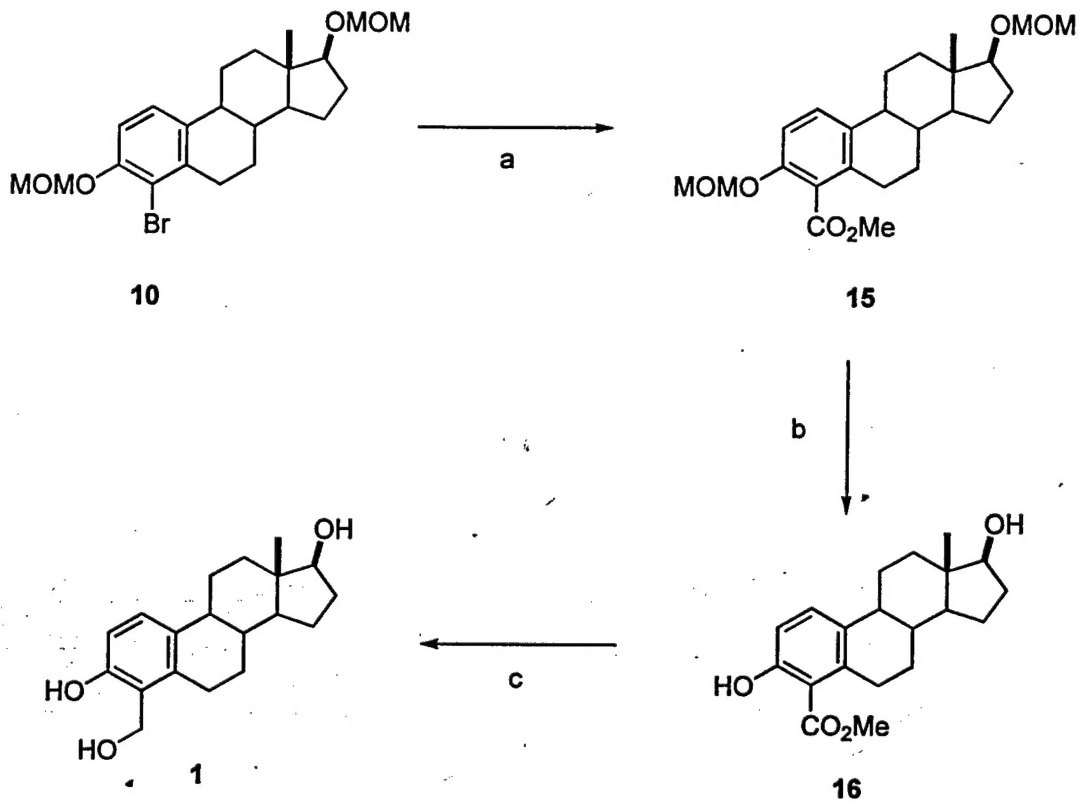
10

Scheme 1



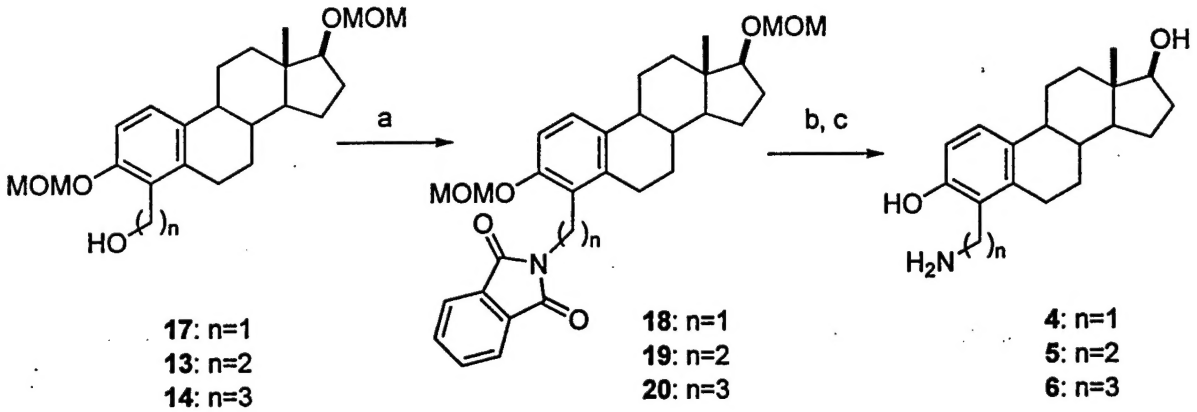
Reagents and conditions: a. N-Bromosuccinimide, EtOH, 54%; b. MOMCl, *i*-Pr_2NEt, THF, Δ, 75%; c. $Pd(PPh_3)_4$, $CH_2{=}CHSnBu_3$, DMF, Δ, 90%; d. $Pd(PPh_3)_4$, $CH_3CH{=}CHSnBu_3$, DMF, Δ, 94%; e. (i) $BH_3{\bullet}THF$, THF, 0 °C, (ii) NaOH, H_2O_2, Δ, **11**→**13** 39%, **12**→**14**, 82%, f. PPTS, MeOH, Δ, **13**→**2** 80%, **14**→**3** 61%.

Scheme 2



Reagents and conditions: a. (i) *n*-BuLi, THF, -78 °C, (ii) CO_2, -78 °C→RT; (iii) CH_2N_2, Et_2O, 0 °C, 76%, b. PPTS, MeOH, Δ, 83%; c. $LiAlH_4$, THF, 0 °C→RT, 51%.

Scheme 3



17: n=1
13: n=2
14: n=3

18: n=1
19: n=2
20: n=3

4: n=1
5: n=2
6: n=3

Reagents and conditions: a. PhthNH, DEAD, Ph_3P, THF; b. NH_2NH_2, EtOH, Δ; c. HCl, MeOH.

Elemental Analysis Data

compound number	chemical formula	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
1	$C_{19}H_{26}O_3 \bullet 0.25H_2O$	74.21	8.59		74.35	8.76	
2	$C_{20}H_{28}O_3 \bullet 0.5H_2O$	73.81	8.98		70.95	9.01	
3	$C_{21}H_{30}O_3 \bullet 0.25H_2O$	75.30	9.17		75.67	8.96	
4	$C_{19}H_{28}NO_2Cl \bullet H_2O$	64.12	8.49	3.94	64.31	8.28	3.94
11	$C_{24}H_{34}O_4$	74.58	8.87		74.24	8.92	
12	$C_{25}H_{36}O_4$	74.96	9.06		74.64	8.83	
13	$C_{24}H_{36}O_5$	71.26	8.97		70.95	9.01	
14	$C_{25}H_{38}O_5$	71.74	9.15		71.40	9.02	
15	$C_{24}H_{34}O_6$	68.88	8.19		68.64	7.86	
16	$C_{20}H_{26}O_4 \bullet 0.5H_2O$	70.71	7.83		70.77	7.83	
17	$C_{23}H_{34}O_5$	70.44	8.75		70.55	8.58	
18	$C_{32}H_{39}NO_6$	72.00	7.37	2.62	71.85	7.40	2.61
19	$C_{31}H_{37}NO_6$	71.65	7.18	2.70	71.44	6.89	2.70
25	$C_{33}H_{41}NO_6$	72.35	7.54	2.55	71.97	7.54	2.60